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## Abstract

Title of Dissertation:

Regulation of innate immune responses is required for *S. mansoni* development

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Helminth blood flukes of the genus *Schistosoma* infect over 200 million people. As a result of host-parasite co-evolution, *S. mansoni* evolved to exploit host immune factors as signals to coordinate its own development. Worms fail to develop normally in RAG<sup>-/-</sup> mice, lacking T and B cells, while development is restored by adoptive transfer of CD4<sup>+</sup> T cells, suggesting that CD4<sup>+</sup> T cells play a central role in regulating parasite development. Recent findings suggest the role of CD4<sup>+</sup> T cells in this process is indirect, being limited to provision of non-cognate T cell help for innate responses which, in turn, facilitate parasite development. In support of this hypothesis, we have found that long-term stimulation of TLR4 by LPS, a pathogen associated molecular pattern (PAMP), in RAG<sup>-/-</sup> mice, in the absence of CD4<sup>+</sup> T cells, also restores worm development, indicating that innate immune

signals are sufficient for parasite development to proceed normally. We have also found that chronic TLR stimulation via other PAMPs that utilize MyD88 dependent signaling, as well as chronic inflammasome signaling were also sufficient to restore worm development in RAG<sup>-/-</sup> mice. Chronic stimulation of both of these pathways resulted in down-regulated pro-inflammatory responses leading us to hypothesize that regulation of innate immune responses are necessary for restoration of *S. mansoni* development. In support of this hypothesis we found blocking TNF- $\alpha$  or administering IL-4 also led to the regulation of pro-inflammatory immune responses that favored parasite development as well. Administering IL-4 to immunodeficient mice also resulted in the establishment of Th2 like immune environment. This suggests that in immunologically intact animals it is the establishment of Th2 responses that promote parasite development. Elucidation of the innate immune signals that control schistosome development could provide leads for the development of new drug targets and vaccine strategies.

**Regulation of innate immune responses is required for *S. mansoni* development**

**By**

**Diana K. Riner**

**Dissertation submitted to the Faculty of the  
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## **Dedication**

I am blessed to come from a long line of strong women. I dedicate this work to my mother Janet Lynn Riner, and my grandmothers Laura Manaci and Joan Riner. From these women I have learned the value of education, hard work, dedication, loyalty, and above all love for family. My mother, who knows me better than I know myself, instilled in all of her children that education was the key to our success and would open doors for us. Grandma Manaci escaped from the extreme poverty of the coal mines and had a life full of struggle and strife yet she never let that steal her goodness or gentle spirit. My Grandma Riner grew up on a farm in southwest Ohio during the depression and remembers having to pluck chickens before school on market day. While she never made me pluck chickens she did pass down to me the same work ethic that was instilled in her and while the farm has long been sold I like to think that its legacy still lives on in her and through her grandchildren.

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**Chapter 1**  
**General Information**

## History and Epidemiology of Schistosomes

### History

Schistosomiasis is the name given to diseases caused by parasitic blood flukes of the genus *Schistosoma*. While there are numerous species of schistosomes, only 5 are considered to be of relevance for human infection and they are *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi*, and *S. intercalatum* [1]. Schistosomes are responsible for two forms of disease - urinary schistosomiasis, whose causative agent is *S. haematobium*, and hepatosplenic schistosomiasis, whose causative agents are *S. mansoni*, *S. japonicum*, *S. mekongi*, and *S. intercalatum* [2]. The three most medically relevant species of schistosomes are *S. mansoni*, *S. haematobium*, *S. japonicum* [2]. Eggs for *S. haematobium* have been found in Egyptian mummies, as have fossil records for its snail intermediate host [3], and the first definitive reports for this disease in Egypt were reported by a French army surgeon during Napoleon's Egyptian campaign in 1798 [1,3].

Theodor Bilharz is credited with discovering *Schistosoma* in 1851, although he named what is now *S. haematobium* as *Distomum haematobium* and credited this species as producing both lateral and terminal spined eggs [1,4]. In 1902, Patrick Mason suggested that the eggs belonged to two distinct schistosomes species, and in 1907 Samson suggesting that lateral-spined eggs should be called *S. mansoni* [1]. Finally in 1915, R.T. Leiper showed these eggs were indeed from two different species of worms, as they required different snail intermediate hosts [1,4,5]. The first report of *S. japonicum* in the modern literature was a description of the ancient disease Katayama fever by Dairo Fujii in 1847 in Japan, with the snail intermediate host being reported in that country in

1913 [5].

## Epidemiology of Schistosomiasis

At present, over 200 million people are infected with schistosomiasis [2,6,7], another approximately 700 million people are at risk of acquiring the infection [8] and the disease is endemic in over 70 countries [7]. However, an estimated 93% of cases worldwide occur in Sub-Saharan Africa, with *S. mansoni* infections responsible for one-third of that number and *S. haematobium* responsible for the other two-thirds [9,10]. After infection, schistosomes are able to survive inside the host for many years despite robust host immune responses [11].

## **Lifecycle of *S. mansoni***

The lifecycle of *S. mansoni* is complicated and involves a definitive mammalian host and a freshwater snail intermediate host of the genus *Biomphalaria*. Adult worms live in the mesenteric venules of their mammalian host, where the females can produce hundreds of eggs per day. These eggs cross through the bowel wall and are excreted in feces. Upon exposure to water, ciliated miracidia emerge from the eggs, penetrate snails of the genus *Biomphalaria*, and develop into cercariae. Cercariae exit the snail host approximately 4 weeks later to find a mammalian host in order to complete development. Humans become infected via contact with water containing cercariae. The cercariae penetrate through the skin, access the bloodstream, travel to the lungs, and then migrate to the liver. At approximately 4 to 6 weeks post infection, female and male worms form

pairs, and migrate to the mesenteric venules, where eggs are released to continue the cycle anew [1,2].

### **Disease pathology associated with *S. mansoni***

#### **Acute Disease**

Infection with *S. mansoni* begins with the penetration of skin by cercariae and can result in a cercarial dermatitis, characterized by formation of a maculopapular rash at the site of penetration as soon as an hour after exposure. This pathology is similar to swimmers itch but less severe [12-15]. Exposure to nonhuman schistosomes, whose hosts are usually birds, results in swimmer's itch that manifests as an intense pruritic dermatitis in response to the dying cercariae [12,14]. The acute phase of *S. mansoni* infection is thought to start around 4 to 6 week post infection (p.i.) and coincides with the beginning of egg laying [11,12,14], although symptoms have been reported as soon as 11 to 21 days post exposure to water containing cercariae [13,16]. Symptoms include fever, malaise, nausea, diarrhea, non-productive cough, headache, edema, and eosinophilia. This is sometimes referred to as Katayama syndrome and is thought to be an immune-mediated hypersensitivity reaction against the migrating parasite or the first egg depositions [2,15,17-19]. Occasionally, acute infection can result in neurological, pulmonary, cardiac, hepatic, or intestinal sequelae. Acute disease usually manifests in individuals experiencing their first infection upon visiting an endemic area [12-14]. Acute disease is not commonly reported in individuals living in endemic areas. Reasons for this include underreporting, due to inadequate healthcare systems, and the early age at

which the primary infection occurs, as individuals in endemic areas have often acquired their first infection by age two [20]. Protection from acute disease is also thought to occur via exposure to schistosome antigens in utero or through breast milk, resulting in an immune response primed to respond to eggs [11,21].

### Chronic disease

Chronic schistosomiasis develops in the months to years following the initial infection and repeated re-infections. Pathology can range from mild intestinal and hepatointestinal changes to severe hepatosplenic pathology [12]. Pathology is caused by parasite eggs that become trapped in the liver, intestinal wall and other tissues [2,22,23].

Granulomas form around the trapped eggs and help control the disease by preventing tissue damage from toxins produced by the eggs [24,25]. However, this granulomatous response can result in fibrosis, leading to intestinal and liver pathology [2,26].

Symptoms for the milder intestinal form of the disease include diarrhea, haematochezia (bloody stool), constipation, and gastric and iliac pain. More severe complications can include colonic stenosis, rectal stenosis, or polyp formation [14,17]. Granuloma formation in the liver can result in excessive deposition of collagen and other extracellular matrix components, leading to periportal liver fibrosis that is sometimes referred to as clay pipe stem fibrosis [2,14,17], and resulting in the occlusion of portal veins[15] that give the liver a “turtle back” appearance [23]. Complications from excessive fibrosis include hepatomegaly, splenomegaly, portal hypertension, ascites, portocaval shunting, and gastrointestinal varices [2,14,15]. Variceal bleeding can result in anemia due to blood loss and severe sudden hemorrhage can result in death [15]. Other

complications from chronic schistosomiasis include pulmonary hypertension [14,27] neuroschistosomiasis, glomerulonephritis, growth retardation in children, as well as possible cognitive and memory impairment in children [2,14,15,17].

## **Treatment**

Praziquantel is the only approved therapy shown to be effective against all species of schistosomes. However, praziquantel is only lethal to adult worms and does not provide protection from juvenile worms or from re-infection. During acute disease, steroids such as prednisone are often used in conjunction with praziquantel to provide relief for symptoms, as treatment with praziquantel alone is often not sufficient [19,28]. Schistosome resistance to praziquantel has also been demonstrated in laboratory settings [29,30] as well as from *S. mansoni* isolates recovered from Kenyan patients [31]. In 2008 the Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) program was enacted via funding from the Bill and Melinda Gates foundation in order to combat schistosomiasis using a variety of strategies, including mass praziquantel administration in numerous counties across Africa (<http://score.uga.edu/>). However, while mass praziquantel administration is helping to reduce the numbers of infections in humans, the disease is not being eradicated in the areas where mass treatments are occurring [6,32,33], raising the concern of rebound mortality if control programs are halted [34]. While important for killing worms and preventing exacerbation of chronic disease, praziquantel will not reverse the chronic pathology that has already occurred. Together these findings highlight why the development of new therapies and vaccines for

treatment and prevention of schistosomiasis is an urgent priority.

## **Vaccine development**

Development of a successful vaccine to combat schistosomiasis has been an unrealized goal in the schistosomiasis community for decades. As natural infection does not eventually confer sterile immunity, two major challenges are (i) how to define protective immunity in humans and (ii) how to engineer a vaccine that generates an immune response to exceed that which is induced by natural infection [35,36]. In light of this, there has been a paradigm shift in regards to schistosome vaccines, from the idea of achieving sterile immunity as is generally the goal of bacterial and viral vaccine candidates, to finding vaccine candidates that decrease worm burdens or fecundity, in the hope of decreasing disease severity when used in combination with chemotherapy [35,37,38]. This is thought to be an achievable goal, as irradiated cercarial vaccines, while not a viable option for human vaccinations, have been shown to confer up to 80% protection in various animal models [39-42] and numerous schistosomal antigens can achieve at least 30% protection [36]. Two promising vaccine candidates are the protein based vaccine candidate fatty acid-binding protein (FABP)-14 (SM14), which has been approved for a phase 1 clinical trial [43], and a DNA-based or recombinant protein vaccine candidate Sm-p80, a protein involved in surface membrane biogenesis [44-46]. Both of these vaccine candidates result in a Th1 biased immune response [21,44-46].

It remains to be determined if Th1 biased immune responses are optimal for generating protection to challenge infections, or if a Th2 biased response would result in

better protection, as the sterile immunity that is seen in rats (a naturally non-permissive host) is associated with the production of IL-4, IL-5 and IL-13 [47]. Sixty percent protection was obtained when mice were vaccinated with the schistosome excretory/secretory (ES) antigens rSG3PDH and TPX MAP along with thymic stromal lymphopoietin (TSLP), a cytokine whose production amplifies Th2 responses [47]. Besides determining the best vaccine candidates to move to clinical trials, challenges remain in determining dosage schedules in coordination with drug treatment, as well as determining measures for reduction of morbidity and mortality to show vaccine efficacy, since sterile immunity is not an achievable aim [35].

### **Immune response to *S. mansoni***

#### **Basic overview**

The immune response to *S. mansoni* occurs in two waves. The first is a T helper 1 response (T<sub>H</sub>1) in reaction to the parasite. During the acute phase, high levels of tumor necrosis factor (TNF) can be measured in the plasma of infected patients and peripheral blood mononuclear cells (PMBCs) produce high levels of TNF, interleukin-1 (IL-1), and IL-6 in reaction to worm antigen [11,16,20]. However, egg production results in a switch from a T<sub>H</sub>1 response to a strong T helper 2 response (T<sub>H</sub>2), characterized by the production of IL-4 and IL-5 [48,49]. Granuloma formation around the eggs is the key to host survival [50,51], with lack of IL-4 leading to a Th1 pro-inflammatory response and resulting in severe disease and early mortality [25,52-55]. Overall, a protective immune response to schistosomes is characterized by an early Th1 response, with production of

IL-2, IFN- $\gamma$ , and TNF- $\alpha$  before egg deposition, then by a switch to a Th2 response, with production of IL-4, IL-5, IL-10, and IL-13 after egg deposition, and then followed by a regulatory response characterized by IL-10 production and downmodulated T-cell responses [56].

Th1 versus Th2 responses during *S. mansoni* infection: The need for a balanced response

In mouse models, protection induced by experimental vaccines correlate with strong Th1 responses directed against the schistosomula stage in the skin and lung [57,58]. However, severe human hepatosplenic disease also correlates with strong Th1 responses directed towards the eggs [59,60]. By 7 to 8 weeks post infection, mouse strains such as C3H or CBA are designated high pathology strains, due to increased parenchymal inflammation, larger poorly circumscribed granulomas and increased splenomegaly. In contrast, low pathology mouse strains such as BL/6 and BALB/c show little parenchymal inflammation, smaller well circumscribed granulomas and more moderate splenomegaly [56]. The very different responses seen in these mouse models can be attributed to CD4 $^{+}$  T cell responses to egg antigens, with Th1 cytokine production predominating in the high pathology mouse strains [61,62]. Lack of IL-4 during *S. mansoni* infection leads to a Th1 pro-inflammatory response and results in severe disease and early mortality [25,52-55]. IL-10 is critical for balancing Th1 versus Th2 responses towards parasite eggs, as IL-10 knockout mice develop a nonpolarized Th1/Th2 immune response that resulted in increased egg granuloma size [54,63]. IL-10/IL-4 deficient mice develop a polarized Th1 response that results in 100% mortality by nine weeks p.i. due in

part to hepatotoxicity[54]. On the other hand IL-10/IL-12 deficient mice, while surviving past week nine p.i., suffered from a progressive wasting disease due to increased granuloma size and fibrosis - a result of a polarized Th2 response characterized by high levels of IL-4 and IL-13 that led to significant mortality in the chronic stages of infection [54]. The increased fibrosis and mortality in these animals is due to IL-13 [53], highlighting the mechanisms by which Th2 responses can detrimental to the host during chronic infection and underscoring the importance of a balanced immune response.

#### Shistosomula influence the development of Type 2 responses

While penetration and invasion of the skin by *S. mansoni* results in upregulation of pro-inflammatory signals, including TNF [64,65], these responses are short lived, with the parasite utilizing excretory/secretory (ES) molecules to modulate initial immune responses. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) are two well characterized ES components [65-68] whose production leads to decreased inflammatory cell recruitment and delayed immune responses in the skin after infection. *S. mansoni* PGE<sub>2</sub> mediates its effects by induced human keratinocytes to produce both PGE<sub>2</sub> and IL-10 [66]. *S. mansoni* PGD<sub>2</sub> mediates its effects by the inhibition of Langerhans cell (LC) migration out of the skin. Other ES products have similar effects, such as inducing apoptosis of T-cells [69] or inhibiting TLR signaling [70]. These early immune modulations by the parasite have a profound effect on parasite survival, as demonstrated by studies of irradiated cercarial vaccine. Irradiated cercariae have delayed migration out of the skin and are unable to migrate out of the lungs [71,72]. The immune response

generated against them also differs from that of normal cercariae, being predominantly Th1 driven and characterized by IFN- $\gamma$  production by T-cells [58,73-75] and IL-12 production by antigen presenting cells [57,76]. Irradiated cercariae based vaccination can confer protection ranging from 60 to 90% [41,42,58].

Worms influence development of Type 2 egg responses

While egg antigens are a critical component in the generation of protective Th2 responses, the early immune response to the developing worm also plays a vital role in the shaping of this immune response. The generation of these Th2 responses has been shown to occur early in *S. mansoni* infection, before the deposition of eggs, and is characterized by the production of IL-4 in response to worm antigen [77,78]. Furthermore, it has been demonstrated that the immune response must first be primed by the developing worms in order for proper Th2 granuloma formation to occur, and that when eggs are injected into naïve animals, the granulomas that form in the liver are much smaller and more abscess-like in appearance [79]. Taken together, these data highlight the importance of immune priming by worm antigens before the appearance of eggs.

Eggs and regulation

Macrophages are of critical importance in the regulation of excessive egg-induced inflammation. Macrophages are broadly defined as either being classically activated by IFN- $\gamma$  (M1), or alternatively activated by IL-4 and IL-13 (M2) [80]. M2 macrophages are

characterized by the expression of YM1, Fizz1 (also known as RELM- $\alpha$ ), and arginase 1 [81,82]. The lack AAMs during acute infection is lethal, as was shown when mice engineered to have macrophages lacking IL-4Ra were infected with *S.mansoni* [83]. Lethality was associated with excessive Th1 responses, resulting in impaired excretion of eggs and leading to increased intestinal pathology and sepsis [83]. Excessive Th2 responses to eggs, on the other hand, are also pathogenic. Deletion of the arginase 1 gene from macrophages results in increased Th2-driven fibrosis during experimental *S. mansoni* infection, highlighting the role of AAMs in not only mediating Th2 inflammation but also in regulating it [84].

**Immune system modulation of *S. mansoni* development is dependent on  
CD4 $^{+}$  T cells**

*S. mansoni*, like many helminths, is able to survive in the human host over long periods of time. The first evidence that immune responses were important in schistosome development came from the administration of corticosteroids during early *S. mansoni* infection in 1957. The author found that administering cortisone acetate resulted in a decreased worm burden [85] in mice receiving the drug therapy. These results were confirmed in 1960 by Weinmann and Hunter, with the authors observing that for administration of cortisone acetate to have an impact on worm burden, it needed to be administered early in infection, as administration at the start of the fourth and fifth weeks of infection had no impact on worm burden [86]. Later, it was shown that the mechanism by which corticosteroids exerted their effects was by immunosuppression and not by

direct toxicity to the worms. These authors also reported that T-cell depletion via thymectomy resulted in a reduced worm burden and resulted in delays in oviposition by the parasites [87,88]. The importance of T cell responses in facilitating *S. mansoni* life cycle completion was also shown by the dependence of the parasite on T cell driven granulomatous inflammation for the excretion of parasite eggs, with the authors suggesting that *S. mansoni* was utilizing the definitive host's immune responses in order to continue its own transmission [87]. Davies (2001) went on to demonstrate that *S. mansoni*, in order to complete development, needed immune signals from its mammalian host. Worms isolated from recombination activating gene deficient (RAG<sup>-/-</sup>) C57BL/6 mice, lacking T and B cells [89], were smaller, did not form as many pairs, and had severely impaired egg production. Worm development could be restored in RAG<sup>-/-</sup> mice by reconstitution with CD4<sup>+</sup> T-cells. Reconstitution with both CD8<sup>+</sup> and CD19<sup>+</sup> B cells failed to restore worm development [90]. Additionally, the need for CD4<sup>+</sup> T cells in order to complete development is evolutionarily conserved among the three medically important species of schistosomes [91].

***S. mansoni* development is not restored as a result of antigen specific  
CD4<sup>+</sup> T cell responses**

Restoration of worm development is not due to schistosome antigen-mediated T cell activation. Worm development was normal in both Bcl10<sup>-/-</sup>deficient and Protein kinase C θ (PKCθ) -deficient mice that possessed T cells incapable of responding to antigen via MHC presentation. In addition, *S. mansoni*-infected transgenic RAG<sup>-/-</sup> mice

with CD4<sup>+</sup> T cell populations specific for single irrelevant antigens (either to chicken ovalbumin (OVA) or pigeon cytochrome C (PCC)), and therefore incapable of responding to schistosome antigen, also exhibited at least partial restoration of worm development [92]. These results suggested that the role of naïve T cells in parasite development was an indirect one and did not involve direct recognition of the parasite. In support of this idea, Lamb demonstrated that there were profound differences in mononuclear phagocyte populations between RAG<sup>-/-</sup> and RAG<sup>+/+</sup> OT-II mice. Possession of naïve T-cells bearing irrelevant TCRs by RAG<sup>-/-</sup> OT-II mice resulted in alteration of mononuclear phagocyte development and promoted a more mature phenotype when compared to RAG<sup>+/+</sup> mice [92].

### **Innate immune signals are sufficient for parasite development to proceed normally**

As parasite development in RAG<sup>-/-</sup>OT-II mice correlated with steady state changes in mononuclear phagocyte development, Lamb hypothesized that direct stimulation of the innate system in RAG<sup>-/-</sup> mice during prepatent infection would restore worm development in those animals. Indeed, she found that administration of LPS, a pathogen associated molecular pattern (PAMP) and toll-like receptor 4 (TLR)-4 ligand [93], to RAG<sup>-/-</sup> mice restored worm development in the absence of CD4<sup>+</sup> T cells [92]. These results raised two important questions that formed the basis of this thesis research. First, we wished to answer the question of how innate immune responses were activated during the course of a natural schistosome infection, in order to provide conditions appropriate for schistosome development. Second, we wished to determine what types of

innate responses and immune factors were required for parasite development to occur.

### **Rationale for Specific Aims**

Innate immune responses are a broad category of responses involving a variety of cell types, pattern-recognition receptors, and signaling pathways. As these categories of responses are so broad, we decided to first focus on LPS and TLR4 signaling, as these had been shown to promote parasite development in RAG<sup>-/-</sup> mice [92]. TLR4 is unique amongst TLRs in that it utilizes both MyD88 and TRIF dependent signaling [94]. we therefore sought to determine which of these pathways was implicated in the restoration of parasite development. Signaling through the MyD88 dependent pathway results in the production of inflammatory cytokines. Signaling via TRIF, results in the expression of IFN-inducible genes leading to the production of type 1 interferons [95]. Determination of the signal adaptor molecules necessary for restoration of worm growth by TLR signaling could clarify which inflammatory molecules are important in worm development. We also wanted to determine if other TLR ligands that shared similar signaling pathways to TLR4 could also restore parasite development in RAG<sup>-/-</sup> mice. Signaling pathways activated via cell surface or endosomal TLR ligation by bacterial, viral, yeast, or protozoan PAMPs are well characterized in the literature and individual PAMP/TLR interactions result in distinct cytokine profiles [95-97]. Determining other PAMP/TLR interactions capable of restoring parasite development would thus assist in clarifying the type of innate inflammatory responses conducive to parasite development.

Bacterial, viral, protozoan, or yeast PAMPs typically would not be present in high

enough quantities to facilitate schistosome development during a normal infection. Furthermore while schistosome egg antigens have been shown to activate TLR signaling, for example lysophosphatidylserine activates TLR2 [98] and lacto-N-fucopentaose III activates TLR4 [99], these antigens would also not be present during parasite development *S. mansoni* 0-to-3-h released larval preparation (0-3hRP) has been shown to stimulate cytokine production by TLR-4 dependent mechanisms, but no specific antigen in the preparation was directly implicated as a TLR4 ligand. The authors speculated that the antigen involved was likely a glycan [100]. Therefore while TLR activation by schistosome antigens is possible, other mechanisms could be responsible for innate immune activation during schistosome infection. An obvious mechanism by which schistosomes can activate innate immune responses is via the tissue damage that occurs as a result of the migratory behavior of the developing parasites [101]. Development of the parasites in portal venules results in liver inflammation and coagulative necrosis before the deposition of eggs begins [102,103]. Necrotic cell death results in the release of damage associated molecular patterns (DAMPs) [104] and robust innate immune responses [105]. Release of DAMPS such as ATP and monosodium urate (MSU) from injured or dying cells results in the activation of the Nalp3 inflammasome, followed by the cleavage of pro-IL-1 $\beta$  to IL-1 $\beta$  by Caspase-1 [106,107]. Schistosome egg antigens have been shown to interact with dectin-2, resulting in Nalp3 activation and release of IL-1 $\beta$  [108] raising the possibility that worm antigens might do the same. Furthermore, malarial hemozoin has been shown in vivo to activate the Nalp3 inflammasome. Schistosomes also release hemozoin as a by-product of red blood cell digestion [109,110] and this could possibly result in Nalp3 inflammasome activation as well, making

inflammasome activation and IL-1 signaling attractive candidates for early innate immune activation.

### **General Hypothesis**

*S. mansoni* development in the definitive mammalian host requires activation of the innate immune response.

### **Specific Aims**

#### Specific Aim I

We hypothesize that PAMP/PRR signaling provides an environment permissive to schistosome development.

Aim I: Determine the signaling pathway by which TLR engagement restores *S. mansoni* development in RAG<sup>-/-</sup> mice.

Sub aim 1: Determine if chronic administration of bacterial or viral PAMPs will restore *S. mansoni* development in RAG<sup>-/-</sup> mice.

Sub aim 2: Determine if chronic cell surface or intracellular TLR signaling will restore *S. mansoni* development in RAG<sup>-/-</sup> mice.

Sub aim 3: Determine if Myd88- or TRIF-dependent signaling will restore *S. mansoni*

development in RAG<sup>-/-</sup> mice.

### Specific aim II

We hypothesize that inflammasome signaling induced in response to tissue injury creates an environment permissive to parasite development.

Aim 2: Determine if danger associated molecular patterns (DAMPs), are important for the restoration of *S. mansoni* development in RAG<sup>-/-</sup> mice.

Sub-aim 1: Characterize early hepatocyte cell death after infection with *S. mansoni* in wild type and RAG<sup>-/-</sup> mice.

Sub-aim 2: To induce hepatocyte death in *S. mansoni* infected RAG<sup>-/-</sup> mice during prepatent infection and examine the role of DAMPS in parasite development.

Sub-aim 3: Administer exogenous DAMPs during *S. mansoni* infection in RAG<sup>-/-</sup> mice and examine the role of DAMPS in parasite development.

### Specific Aim III

We hypothesize IL-1/IL-1R signaling is a critical contributor to the permissive environment necessary for schistosome development.

Aim 3: Determine role of IL-1/IL-1R signaling in restoration of *S. mansoni* development

in immunodeficient mice.

Sub-aim 1: Breed RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice in order to determine the role IL-1R signaling plays in restoration of parasite development.

Sub-aim 2: Examine the role of IL-1 $\beta$  and TNF- $\alpha$  in restoration of parasite development in immunodeficient mice.

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## **Chapter 2**

**Regulation of pro-inflammatory responses during pre-patent schistosome  
infection creates an immune environment permissive for parasite development**

## Abstract

Helminth blood flukes of the genus *Schistosoma* infect over 200 million people. As a result of host-parasite co-evolution, *S. mansoni* evolved to exploit host immune factors as signals to coordinate its own development. Worms fail to develop normally in RAG<sup>-/-</sup> mice, lacking T and B cells, while development is restored by adoptive transfer of CD4<sup>+</sup> T cells, suggesting that CD4<sup>+</sup> T cells play a central role in regulating parasite development. Recent findings suggest the role of CD4<sup>+</sup> T cells in this process is indirect, being limited to provision of non-cognate T cell help for innate responses which, in turn, facilitate parasite development. In support of this hypothesis, we have found that long-term administration of LPS to RAG<sup>-/-</sup> mice, in the absence of CD4<sup>+</sup> T cells, also restores worm development, indicating that innate immune signals are sufficient for parasite development to proceed normally. LPS activates TLR-4 resulting in production of TNF- $\alpha$  and IL-1 $\beta$ . However, chronic administration of LPS down-regulates the pro-inflammatory response leading us to hypothesize that regulation of innate immune responses are necessary for restoration of *S. mansoni* development. In support of this hypothesis we found blocking TNF- $\alpha$  or administering IL-4 resulted in decreased expression of IL-1 $\beta$  in *S. mansoni* infected RAG<sup>-/-</sup> mice, as well as, restored parasite development. Current research efforts are focused on how regulation of IL-1 $\beta$  influences schistosome development. Elucidation of the innate immune signals that control schistosome development could provide leads for the development of new drug targets and vaccine strategies.

## Introduction

As a result of extensive host-parasite co-evolution, helminths exploit resources within their hosts to complete their development and ensure transmission to new hosts. Indeed, most helminths are obligate parasites, requiring the intra-host environment for successful life cycle completion. However, for the most part, the precise host factors that helminths require or utilize, in terms of host cells or molecules, are poorly defined. Previously, CD4<sup>+</sup> T cells were shown to play a fundamental role in schistosome development [1-3], with significant impairment of parasite growth and reproductive activity occurring in the absence of CD4<sup>+</sup> T cells. While the precise mechanism by which CD4<sup>+</sup> T-cells mediate this effect is unclear, the mechanism is indirect, as chronic stimulation of innate immune responses with LPS, a toll-like receptor 4 (TLR4) agonist, during pre-patent infection was able to restore parasite development in the absence of CD4<sup>+</sup> T cells [4]. Thus, all the host factors necessary for schistosome development are present, or at least can be induced, independently of CD4<sup>+</sup> T cells. However, whether the mechanisms by which CD4<sup>+</sup> T cells and chronic LPS stimulation restore schistosome development share any common elements has remained an open question.

Regulation of proinflammatory responses is critical for control of *S. mansoni* infections [5] as well as survival from gram negative sepsis [6]. In the case of schistosomes, and other helminths in general, infection results in the establishment of robust Th2 responses that modulate pro-inflammatory processes [7,8]. In schistosomiasis, Th2 responses against parasite antigens are required for the formation of protective

granulomas around parasite eggs [9,10]. Th2 responses to worm antigens develop even before the onset of egg production [11,12] and there is evidence that this immune priming by the developing worms is necessary to ensure proper Th2 granuloma formation [13]. Th2 responses are also critical for host survival after egg production begins, as lack of IL-4 signaling leads to severe disease and early mortality as a result of excessive pro-inflammatory processes [9,10,14-16]. Thus, in schistosomiasis, Th2 responses serve a dual purpose, to mediate granuloma formation and to regulate inflammation.

Here, we present evidence to suggest that, while fundamentally different, chronic exposure of immunodeficient mice to inflammatory stimuli and CD4<sup>+</sup> T cells in immunocompetent mice ultimately promote parasite development by resulting in a similar outcome, namely the establishment of an immunological milieu where inflammatory processes are regulated. These findings provide insights into the developmental requirements of schistosomes and may identify host dependencies that could be exploited to disrupt schistosome infection.

## Materials and Methods

Ethics statement:

All animal studies were conducted in accordance with established protocols approved by the USUHS Institutional Animal Care and Use Committee.

Experimental mice:

RAG-1<sup>-/-</sup> mice on a C57BL/6 background were originally purchased from Jackson laboratory (Bar Harbor, ME) and then bred in-house for experimental use. C57BL/6 mice were purchased from the National cancer institute (NCI, Frederick, MD). RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> were generated by crossing C57BL/6 RAG-1<sup>-/-</sup> to C57BL/6 IL-1R<sup>-/-</sup> mice purchased from Jackson laboratory (Bar Harbor, ME). The RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> genotype was confirmed via PCR. All mice used in experiments were age matched.

Parasite parameters:

Mice were infected percutaneously via tail exposure to water containing 160 *S. mansoni* cercariae (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails. At 6 weeks post infection (p.i.) mice were sacrificed. Worms were perfused from the portal system and immediately fixed in 4% neutral buffered formaldehyde. Male and female worms were counted and photographed at 20x magnification using a Nikon D80

10.0 megapixel digital camera attached to a Zeiss trinocular dissecting microscope. Worm growth was assessed by measuring the length of male worms from digital micrographs Image J software (<http://rsb.info.nih.gov/ij>), as described previously[4]. Only male worms were measured as female growth is dependent upon receiving developmental cues from pairing with maturing males [17]. Sexual maturation of the parasites was assessed by calculating egg production per worm pair from liver egg burdens, as described previously[4]. Briefly, liver tissue was homogenized and digested in 0.7% trypsin phosphate buffered saline (PBS) solution and the released eggs were counted under a dissecting microscope.

Measuring areas of liver inflammation and coagulative necrosis:

C57BL/6 wild type and RAG-1<sup>-/-</sup> mice were infected with cercariae as described above. At 4 weeks p.i., mice were sacrificed and their livers removed and immediately fixed in 35 ml of 4% neutral buffered formaldehyde. Liver sections were cut and stained with hematoxylin and eosin stain (H&E stain, Histoserv INC., Germantown, MD). Slides were digitally scanned using the Hamamatsu Nanozoomer 2.0RS (Hamamatsu City, Japan). Tissue sections were analyzed using the Nanozoomer digital pathology (NDP) software. At low magnification, random areas of liver tissue measuring 20 mm<sup>2</sup> in total were selected. The selected sections were then scanned at 5X magnification and areas of inflammation were measured. For each liver, sections were obtained at 3 different levels and measurements were taken for at least 3 different tissue sections. The percentage area of inflammation was determined by summing up the area occupied by inflammatory

infiltrate and dividing it by the total area examined. The percentage area occupied by coagulative necrosis was determined by the same method.

Acetaminophen (AAP) and D-(+)-galactosamine hydrochloride (GalN) treatment:

RAG-1<sup>-/-</sup> mice were infected with cercariae as described above. Mice received weekly intraperitoneal (i.p.) injections of AAP (Sigma-Aldrich, St Louis, MO) at a dose of 5 mg/mouse dissolved in 100 µl 30% DMSO for the first 3 weeks. Mice then received AAP at a dose of 10 mg/mouse dissolved in 30% DMSO for the remaining 3 weeks. Control mice received weekly i.p. injections of 30% DMSO. D-GalN (MP Biomedicals, Solon, OH) -treated mice received biweekly i.p. injections at a dose of 10mg/mouse for 6 weeks, using PBS without calcium and magnesium as a vehicle. Control mice received biweekly i.p. injections of PBS (Mediatech, Manassas, VA) alone. At 6 weeks p.i. mice were sacrificed, H&E staining of livers was performed to confirm liver inflammation in treated mice, and parasite parameters were determined as described above.

Monosodium urate (MSU), Alum,LPS, and Poly-IC treatment:

RAG-1<sup>-/-</sup> mice were infected with cercariae as described above. Mice received biweekly i.p. injections of MSU (Invivogen, San Diego, CA) at a dose of 500 µg/mouse, Imject Alum (Thermo Scientific, Rockford, IL) at a dose of 1 mg/mouse, ultrapure LPS, *E.coli* 0111:B4 (Invivogen) at a dose of 20 µg/mouse, or Poly-IC 20 µg or 40 µg /mouse. Control mice received biweekly i.p. injections of PBS without calcium and magnesium.

At 6 weeks p.i. mice were sacrificed and parasite parameters were determined as described above.

#### Anti-TNF- $\alpha$ treatment:

RAG-1<sup>-/-</sup> mice were infected with cercariae as previously described. Mice received weekly i.p. injections of Adalimumab (Abbott, Chicago, IL) at a dose of 100  $\mu$ g/mouse, using PBS without calcium and magnesium as a vehicle. Control mice received weekly i.p. injections of PBS alone. At 6 weeks p.i., mice were sacrificed and parasite parameters were determined as described above.

#### IL-4 complex treatment

RAG-1<sup>-/-</sup> mice were infected with cercariae as described above. Mice received weekly i.p. injections of 5  $\mu$ g IL-4 (Peprotech, Rocky Hill, New Jersey) complexed to 25  $\mu$ g anti-IL-4 antibody 11B11 (BioXCell, West New Lebanon, New Hampshire) (Jenkins, 2011). Control mice received weekly i.p. injections of the isotype control antibody HRPN (BioXCell). At 6 weeks p.i. mice were sacrificed, H&E staining of livers was performed to confirm liver inflammation in treated mice, and parasite parameters were determined as described above.

#### RNA isolation, purification, and Real time PCR

RNA was isolated from the spleens and/or livers of wild type or RAG-1<sup>-/-</sup> mice. After removal, tissues were immediately placed in 1 ml RNA-BEE (Tel-Test, Friendswood, Texas), homogenized, snap-frozen in liquid nitrogen and stored at -80°C until isolation of total RNA, following manufacturer's instructions. RNA was further purified following the RNeasy mini protocol for RNA cleanup (Qiagen, Valencia, California). Purified RNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). 2 ug of RNA was used for cDNA preparation using a high capacity RNA to cDNA kit (Invitrogen, Grand Island, New York) following manufacturer's instructions. Real time PCR was performed with a MJ Research Chromo4 PTC-200 thermocycler unit (Bio-Rad, Hercules, CA) using Taqman gene expression assays and TaqMan gene expression master mix (Invitrogen) following manufacturer's instructions. Assays for the following mRNAs were performed: *rsp29*, GAPDH, IL-1 $\beta$ , TNF- $\alpha$ , CCL2, Relm- $\alpha$ , and YM1. Expression of genes of interest was normalized to the expression of GAPDH or *rsp29* and fold changes in expression were calculated following the  $2^{-\Delta\Delta CT}$  method [18].

### Statistical analysis

All statistical analyses were performed using GraphPad Prism Inc. version 4.0 software (San Diego, California). Significant differences between two groups were determined using a student's unpaired T-test with Welch's correction or a Mann-Whitney test. Significant differences between 3 or more groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. *P* values of less than 0.05

were considered significant. 3-5 mice were used per experimental group and each experiment was repeated at least twice.

## Results

*Pre-patent *S. mansoni* infection fails to induce liver inflammation and necrosis in immunodeficient mice*

Although chronic lipopolysaccharide stimulation, administered twice weekly during the first six weeks of infection, can restore schistosome development in RAG<sup>-/-</sup> mice, this stimulus is not present at high concentrations in mouse plasma. We therefore sought to identify other inflammatory processes occurring during the pre-patent stage of schistosome infection in normal mice, but not in RAG<sup>-/-</sup> mice, as these might be candidates for a physiological stimulus for parasite development. Before the deposition of eggs, worm development in the portal vasculature is associated with both liver inflammation and hepatocellular necrosis of an unknown etiology [19,20]. To determine whether this pathology also occurs in RAG<sup>-/-</sup> mice, we compared liver tissue sections from 4 week-infected RAG<sup>-/-</sup> and wild type mice. As previously reported [19,20], wild type mice exhibited areas of coagulative necrosis and infiltration with inflammatory cells (Figure 1A). Inflammatory infiltrates, consisting of lymphocytes, eosinophils and mononuclear cells, were located in periportal areas and in the parenchyma, and also surrounded necrotic areas (Figure 1A). In contrast, areas of coagulative necrosis were not seen in the 4 week-infected RAG<sup>-/-</sup> mice (Figure 1B), with the exception of two animals where small foci of necrosis were detected (data points included in Figure 1C). Furthermore, we observed very little liver inflammation in 4 week-infected RAG<sup>-/-</sup> mice (Figure 1B), which, when present, was restricted to areas around or near vessel walls

(figure 1B). The mean percentage area occupied by necrotic liver tissue was 1.6 % for wild type animals, while RAG<sup>-/-</sup> mice exhibited almost none (Figure 1C). Likewise, the mean percentage area occupied by inflammatory infiltrates was 8.7 % in wild type mice, while RAG<sup>-/-</sup> mice exhibited almost none (Figure 1D). These data show that failure of parasite development in RAG<sup>-/-</sup> mice correlates with a lack of liver necrosis and inflammation. Furthermore, while the etiology of the liver necrosis in wild type mice is unknown, these data suggest that death of hepatocytes during pre-patent schistosome infection requires an intact adaptive immune system.

*Endogenous danger-associated molecular patterns restore S. mansoni development in immunodeficient mice.*

In view of the fact that a failure of parasite development in RAG<sup>-/-</sup> mice correlates with a lack of liver necrosis and inflammation, we hypothesized that induction of liver necrosis and inflammation would restore parasite development in these animals. To test this hypothesis, we administered hepatotoxins (either acetaminophen or D-galactosamine) to RAG<sup>-/-</sup> mice throughout pre-patent infection, at doses sufficient to result in hepatocellular death and inflammation [21-24], in an attempt to simulate the cell death observed in wild type mice. While chronic hepatotoxin treatment did not recapitulate the coagulative necrosis seen in wild type mice, both treatments induced histological evidence of widespread hepatocellular damage (data not shown). Furthermore, both treatments partially restored parasite growth when compared to control

RAG<sup>-/-</sup> animals that received vehicle alone (figure 2A and 2B), while parasite egg production was unaffected (data not shown).

Cellular injury results in the release of uric acid into the extracellular environment [25-28] that crystallizes to form monosodium urate (MSU) [29,30], an endogenous DAMP that activates the NALP3 inflammasome [31]. Since chronic hepatotoxin treatment partially restored parasite development in RAG<sup>-/-</sup> mice, we hypothesized that DAMP-mediated inflammatory processes would also restore parasite development in these animals. To test this hypothesis, we administered MSU to infected RAG<sup>-/-</sup> mice throughout the first six weeks of infection and compared worm development to that in control RAG<sup>-/-</sup> mice that received vehicle alone. Treatment with MSU resulted in robust restoration of parasite growth (Figure 2C) and egg production (Figure 2D) in RAG<sup>-/-</sup> mice suggesting that, like LPS, chronic DAMP-mediated inflammation can also stimulate parasite development. In further support of a role for inflammasome-mediated inflammation in stimulating parasite development, we also found that treatment with alum, an exogenous NALP3 inflammasome agonist [32,33], also resulted in robust restoration of parasite growth (Figure 2E) and egg production (Figure 2F) in RAG<sup>-/-</sup> mice. Taken together, these data suggest that, like the exogenous danger signal LPS, endogenous danger signals that stimulate inflammation via inflammasomes can also stimulate schistosome development.

*Parasite development correlates with regulation of IL-1 $\beta$  transcription*

By serving as a molecular platform for caspase 1 activation, inflammasomes drive IL-1 $\beta$ -mediated inflammation by catalyzing the conversion of inactive pro-IL-1 $\beta$  to the bioactive form. As two different inflammasome agonists restored parasite development in RAG $^{-/-}$  mice, we hypothesized that IL-1 $\beta$ -mediated inflammation may be implicated in controlling parasite development. To address this issue, we first examined IL-1 $\beta$  mRNA levels during pre-patent infection of wild type mice. Unexpectedly, we found that steady-state splenic mRNA levels of IL-1 $\beta$  in wild type mice at 3 and 4 weeks p.i. were downregulated compared to the baseline levels found in non-infected control mice (Figure 3A). In contrast, IL-1 $\beta$  mRNA levels remained unchanged in the spleens of 4 week-infected RAG $^{-/-}$  mice when compared to non-infected controls (Figure 3B). Thus, normal parasite development correlated with downregulation of steady-state IL-1 $\beta$  transcription. To test whether a failure to downregulate IL-1 $\beta$  signaling in RAG $^{-/-}$  mice is the cause of impaired schistosome development in these animals, we infected RAG $^{-/-}$  IL-1R $^{-/-}$  knockout mice, predicting that, if this were the case, ablation of IL-1 signaling would restore parasite development in a RAG-deficient context. However, worms recovered from RAG $^{-/-}$  IL-1R $^{-/-}$  mice 6 weeks p.i. did not differ significantly from those obtained from RAG $^{-/-}$  mice, being small in size (Figure 3C) and reproductively inactive (Figure 3D). Therefore, parasite development correlates with regulation of IL-1 $\beta$  transcription, but IL-1R signaling is not directly responsible for inhibiting parasite development in RAG $^{-/-}$  mice.

*Chronic innate immune stimulation with LPS or MSU results in downregulation of proinflammatory signals*

As our examination of IL-1 $\beta$  transcription in wild type and RAG $^{-/-}$  mice revealed that normal schistsosome development correlated with downregulation of IL-1 $\beta$  transcription, we next examined the effect of the LPS and MSU treatment regimens on IL-1 $\beta$  transcription in RAG $^{-/-}$  mice, as both treatments restore parasite development in these animals. Treatment with LPS (Figure 4A) or MSU (Figure 4B) throughout the first six weeks of infection resulted in downregulation of splenic IL-1 $\beta$  mRNA levels in infected RAG $^{-/-}$  mice by week six post infection, similar to the downregulation seen in infected wild type mice (Figure 3A). Thus, while downregulation of IL-1 $\beta$  transcription is mediated by the adaptive immune system in wild type mice, chronic administration of LPS or MSU to RAG $^{-/-}$  mice can also result in IL-1 $\beta$  downregulation, in the absence of an adaptive immune system. Furthermore, chronic LPS and MSU treatments resulted in downregulation of other proinflammatory signals, as evidenced by reduced splenic mRNA levels for TNF (Figure 4C and 4D) and CCL2 (Figure 4E and 4F), a chemokine important for inflammatory macrophage recruitment [34-36]. Finally, we found that transcriptional downregulation of proinflammatory genes to levels lower than those in control animals required chronic exposure to MSU (Supplementary Figure 1 A-C), as transcription of proinflammatory genes peaked rapidly following a single injection of MSU (data not shown) and then returned to the levels observed in non-treated mice by 18 hours post injection (Supplemental Figure 1 D-F).

*Parasites fail to develop when proinflammatory gene transcription is sustained*

Our analysis of wild type, RAG<sup>-/-</sup> and MSU- and LPS-treated RAG<sup>-/-</sup> mice showed that normal parasite development correlates with the overall downregulation of proinflammatory gene transcription. However, we considered the possibility that elevated proinflammatory gene transcription early in the course of LPS or MSU treatment, before regulation was induced, could be the factor stimulating parasite development rather than the ultimately downregulated state. To explore this possibility we sought to identify comparable treatments where chronic administration of an inflammatory stimulus did not result in downregulated proinflammatory gene expression. To this end, we found that administration of poly I:C [37], a TLR3 ligand, to RAG<sup>-/-</sup> mice throughout the first six weeks of infection, resulted in upregulation of splenic mRNA for IL-1 $\beta$ , TNF, and CCL2 (Figure 5 A-C), rather than their downregulation. Consistent with a role for proinflammatory gene downregulation in permitting schistosome development, chronic administration of poly I:C also failed to restore parasite development in RAG<sup>-/-</sup> mice, as worms recovered from treated animals did not differ significantly in size (figure 5D-E) or egg output (data not shown) from vehicle-treated controls at either of the two doses tested.

*TNF blockade restores parasite development in immunodeficient mice*

As restoration of parasite development in RAG<sup>-/-</sup> mice correlated with downregulation of proinflammatory gene transcription (in LPS- and MSU-treated RAG<sup>-/-</sup> mice) and was unaltered when proinflammatory gene transcription was sustained (in poly I:C-treated RAG<sup>-/-</sup> mice), we hypothesized that it was the downregulation of

proinflammatory genes that permits parasite development to proceed. To test this hypothesis, we attempted to suppress proinflammatory gene activity in RAG<sup>-/-</sup> mice by blocking TNF signaling with a neutralizing antibody, which has been shown to decrease IL-1 $\beta$  production in models of sepsis [38-40] and arthritis [41]. Administration of the anti-TNF antibody did not cause acute increases in proinflammatory gene expression (data not shown), and its administration throughout pre-patent infection led to the downregulation of IL-1 $\beta$ , TNF- $\alpha$ , and CCL2 transcription in the spleens of RAG<sup>-/-</sup> mice by four weeks post infection (Figure 6A-C), similar to that observed in wild type mice and with chronic LPS or MSU treatment in RAG<sup>-/-</sup> mice. Furthermore, anti-TNF treatment resulted in significant increases in parasite size (Figure 6D) and reproductive activity (Figure 6E) when compared to control RAG<sup>-/-</sup> mice. Thus, these data supported our hypothesis that schistosome development requires the downregulation of proinflammatory gene transcription during pre-patent infection.

#### *Administration of IL-4 complex restores parasite development in immunodeficient mice*

In *S. mansoni*-infected wild type mice, downregulation of IL-1 $\beta$  transcription occurs via an adaptive immune mechanism, as there is a failure of IL-1 $\beta$  mRNA downregulation when the adaptive immune system is ablated (Figure 3). In previous studies, we showed that, prior to the onset of egg production, pre-patent schistosome infection results in the rapid establishment of a T helper 2 response [11,12], where CD4 $^{+}$  T cells produce significant quantities of IL-4 in response to worm antigens [11]. As IL-4 is a type 2 cytokine that regulates proinflammatory signals, including IL-1 $\beta$  [42-44], we

hypothesized that IL-4 may represent the adaptive mechanism by which IL-1 $\beta$  is regulated in wild type mice. To test whether IL-4 was sufficient to regulate proinflammatory gene transcription in RAG $^{-/-}$  mice, we administered IL-4 complex (IL-4c) to infected RAG $^{-/-}$  mice during pre-patent infection and examined proinflammatory gene transcription in the spleen at week six post infection. Administration of IL-4c resulted in down-regulation of IL-1 $\beta$  and TNF transcription in the spleens of treated RAG $^{-/-}$  mice (Figure 7A and 7B), similar to that observed in wild type mice and RAG $^{-/-}$  mice treated with LPS, MSU or anti-TNF. Transcription of CCL2 was also reduced by IL-4c treatment, although the difference between treated and control animals narrowly avoided attaining statistical significance (Figure 7C). The transcription of RELM- $\alpha$  and YM1, both markers of alternative macrophage activation [45], was dramatically up-regulated in the spleens (data not shown) and the livers of IL-4c-treated animals (Figure 7D and 7E), suggesting that IL-4c treatment induced an innate type 2 response and the accumulation of alternatively activated macrophages in livers of RAG $^{-/-}$  mice. Indeed, administration of IL-4c also restored the accumulation of mononuclear cells in the livers (Figure 7F) of infected RAG $^{-/-}$  mice and induced giant cell formation (Figure 7G), a previously reported hallmark of alternatively activated macrophage responses [46]. Finally, administration of IL-4c resulted in the restoration of parasite growth (Figure 7H) and reproductive activity (Figure 7I). Our data demonstrate that IL-4, a type 2 cytokine produced as part of the adaptive immune response to pre-patent schistosome infection, is sufficient to regulate proinflammatory signals and restore schistosome development and may represent the mechanism by which CD4 $^{+}$  T cells permit normal parasite development in wild type mice.



## Discussion

Numerous lines of evidence indicate that type 2 responses are beneficial in schistosome infection, not because these responses mediate immunity against schistosomes but because they limit potentially damaging proinflammatory responses. For example, in mice deficient in IL-4, IL-4 and IL-10, IL-4 and IL-13 or IL-4 receptor, decreased host survival is observed during acute schistosome infection due to excessive proinflammatory cytokine expression and increased liver and intestinal pathology [9,10,15,47]. Likewise, in schistosomiasis patients, severe disease is correlated with decreased production of type 2 cytokines and elevated levels of IFN- $\gamma$ , TNF and NO [48]. However, there is also evidence that type 2 responses ultimately benefit schistosomes, and that this benefit extends beyond the obvious relationship between extended host survival and the increased likelihood of transmission to snail intermediate hosts. For example, it has long been recognized that egress of schistosome eggs across the bowel wall is immune-dependent [49]. Subsequent macrophage-specific ablation of IL-4R expression showed that IL-4/IL-13-responsive macrophages are specifically required for egg passage into the intestinal lumen. These observations suggest that host-parasite co-evolution has not only selected for immune responses that prolong host survival, but also for parasites that are able to take advantage of the resulting immunological milieu. The data we present here support the hypothesis that control of proinflammatory signals may also be intimately linked to parasite development before the onset of egg production.

Our finding that chronic stimulation with LPS could restore schistosome development in RAG<sup>-/-</sup> mice presented a paradox, as there is no obvious parallel between the inflammatory response to LPS and the response induced by pre-patent schistosome infection in wild type mice. However inflammation, albeit in response to necrotic hepatocytes, is a feature of pre-patent schistosome infection in immune-competent, but not RAG<sup>-/-</sup> mice, suggesting there is a link between inflammation and normal parasite development. In support of this hypothesis, we show here that restoration of DAMP-mediated inflammation in RAG<sup>-/-</sup> mice also restored parasite development. While endogenous DAMPs stimulate inflammation by pathways distinct from exogenous PAMPs such as LPS, our finding that both can restore parasite development suggests it is the inflammation itself rather than the inciting cause that modulates parasite development. The contribution of necrosis-induced inflammation to promoting parasite development in wild type mice is an interesting and unresolved question. One way to address this question may be to inhibit necrosis in wild type mice and examine for effects on parasite development. Identification of the mechanism leading to hepatocellular necrosis in wild type mice may make this approach possible. The absence of necrosis in RAG<sup>-/-</sup> mice suggests that adaptive responses are involved in necrosis induction. Alternatively, the lack of necrosis in these animals may be a result of diminished parasite growth, rather than a cause. However, we have not observed hepatocellular necrosis in RAG<sup>-/-</sup> mice where parasite development is restored by LPS treatment (data not shown), suggesting that developing parasites do not directly cause liver necrosis.

Our observation that steady state transcription of the pro-inflammatory cytokine IL-1 $\beta$  is down-regulated in infected wild type mice, but not in RAG<sup>-/-</sup> animals, further

suggested a role for inflammatory processes in schistosome development, but in an inhibitory capacity. However, the permissiveness of wild type mice for parasite development is not specifically due to downregulation of IL-1 signaling, as ablation of IL-1R activity in a RAG<sup>-/-</sup> context did not restore parasite development. This result led us to hypothesize that parasite development may require the more general regulation of proinflammatory processes that is mediated by type 2 responses, such as those induced by pre-patent schistosome infection. If this hypothesis were correct, we predicted that chronic LPS or MSU administration restored parasite development in RAG<sup>-/-</sup> mice because they also result in regulation of inflammatory signaling. The induction of LPS tolerance in response to repeated LPS exposure is a well-recognized negative feedback mechanism, thought to be mediated by a variety of mechanisms including regulation of downstream protein kinases, resulting in downregulation of inflammatory cytokine transcription [6]. However, we show here that chronic MSU exposure also results in downregulation of proinflammatory signals. While MSU signals via pathways distinct from LPS, the existence of negative feedback mechanisms that limit the damage that may be caused by persistent MSU signaling is not unexpected. Evidence for the induction of anti-inflammatory mechanisms by endogenous DAMPs exists. First, toxicological injury to the liver by AAP first results in an early pro-inflammatory response that is directed by classically activated M1 macrophages, but this initial response is followed by suppression of the initial pro-inflammatory response and promotion of wound healing by immunoregulatory, alternatively activated (M2) macrophages [50,51]. Second, both alum and uric acid have been shown to promote Th2 immunity and suppression of pro-inflammatory processes through NALP3 independent mechanisms [52,53]. Similarly,

LPS tolerance is associated with induction of immunoregulatory M2 macrophages [54].

Based on our findings, we hypothesized that LPS and MSU restored parasite development in RAG<sup>-/-</sup> mice by virtue of their ability to induce regulation of pro-inflammatory signals when administered chronically, thus mimicking the regulation associated with type 2 responses that normally occur during pre-patent infection of wild type mice.

To further test whether inflammation per se, or the regulation that results from the inflammation was required for parasite development, we sought to identify inflammatory stimuli that did not lead to regulation. We found that the TLR3 ligand poly I:C, even when administered repeatedly on the same regimen as LPS or MSU, failed to reduce baseline levels of inflammatory gene transcription, resulting instead in overall elevated levels of transcription, even 18 hours post administration. Unlike other TLR ligands like LPS, which stimulate MyD88-dependent signaling, poly I:C responses are propagated via distinct TRIF-mediated pathways that appear not to be subject to the same negative feedback regulation. Chronic poly I:C administration therefore afforded us the opportunity to examine schistosome development in the context of persistent inflammation without the associated regulation. Consistent with a role for regulation in parasite development rather than inflammation, chronic administration of poly I:C, at two different doses, failed to enhance parasite development.

Because poly I:C signaling is mediated via a distinct receptor and adapter molecule, we cannot exclude the possibility that some essential component of the response induced by MSU or LPS is absent from the response to poly I:C. However, we reasoned that if regulation of inflammation was the critical element in permitting parasite

development, then direct regulation of inflammation in the absence of exogenous inflammatory stimuli would also be able to restore schistosome development. The success of anti-TNF neutralization therapy in controlling inflammatory disorders is due to the ability of this intervention in broadly controlling inflammation, mediated by TNF and associated signals, including IL-1 $\beta$ . In the absence of any additional inflammatory stimuli, administration of anti-TNF antibodies to infected RAG<sup>-/-</sup> mice recapitulated the regulation of inflammatory gene transcription observed after chronic LPS or MSU administration and also restored schistosome development, lending further support to the necessity of regulation for normal parasite development.

In an immunocompetent host, pre-patent schistosome infection induces a Th2 response, characterized by production of IL-4 by CD4 $^{+}$  T cells in response to schistosome antigens. In addition to driving Th2 effector mechanisms such as antibody isotype class switch recombination in B cells and M2 macrophage development, IL-4 also regulates pro-inflammatory signals and is therefore a likely principle regulator of pro-inflammatory processes during pre-patent schistosome infection. We therefore hypothesized that regulation mediated by IL-4 may be the principle contribution of the adaptive response in wild type mice to permitting parasite development. Consistent with this role, IL-4 administration to RAG<sup>-/-</sup> mice was sufficient to restore regulation of IL-1 $\beta$  and TNF transcription and induce type 2 responses, and also restored parasite development. Thus, a single type 2 cytokine was sufficient to significantly augment schistosome growth and egg production. Despite this finding, IL-4 is unlikely to be the only host factor to promote schistosome development, as parasite development proceeds normally when IL-4 signaling is blocked in otherwise immunologically intact mice, whether by anti-IL-4

antibody or through genetic disruption. We therefore conclude there is likely considerable redundancy in the requirement of schistosomes for regulation, as illustrated by the diversity of mechanisms by which parasite development can be restored in RAG<sup>-/-</sup> mice (chronic administration of LPS or MSU, or administration to anti-TNF antibody or IL-4). These findings also suggest that the host elements required for schistosome development may be common components to the responses induced by each of these mediators. For example, development of alternatively activated M2 macrophages rather than classically activated M1 macrophages is promoted in each of these immunological situations [54-59]. Thus M2 macrophages are one possible host factor that schistosomes co-opt to complete development. There is already evidence for schistosome dependence on M2 macrophages later in infection, as these cells are critical to the egress of schistosome eggs from the body of the host. As M2 macrophages are a specific hallmark of the host response to schistosomes and other helminths, the hypothesis that schistosomes have evolved to specifically exploit this aspect of the host response is an attractive one.

Why regulation of pro-inflammatory signals would influence schistosome development remains to be determined. Allen and Wynn recently suggested that Th2 immunity evolved in order to supply a fast response and to repair the tissue damage generated by helminthes[60] , highlighting that the need for regulation of innate immune responses is not limited to schistosome infections but is a feature of many immune responses directed towards tissue-penetrating helminths. For example, M2 macrophages and the generation of Th2 responses are critical for limiting lung tissue damage after experimental *N. brasiliensis* infection [61], by controlling initial IL-17-driven inflammatory responses and promoting resolution of tissue damage [62]. AAMs have

also been shown to limit brain tissue pathology in a murine model for neurocysticercosis [63]. Both STAT6-/- mice [64] and TLR2-/- [63] mice had decreased numbers of AAMs found in brains containing *Mesocestoides corti* cysts compared to infected wild type controls resulting in increased parasite burden and disease severity. The generation of Th2 responses has been shown to occur early in *S. mansoni* infection [11,12] and is critical for host survival after egg production begins [9,10,14-16]. Macrophages particularly alternatively activated macrophages are of critical importance in the regulation of excessive egg-induced inflammation and the lack AAMs during acute infection is lethal, as shown by the macrophage-specific ablation of IL-4R $\alpha$  expression [47]. How type 2 responses contribute to schistosome development remains unclear. As mediators of tissue repair and remodeling, one possibility is that M2 cells mediate critical niche remodeling in portal venules where the rapidly growing schistosomes reside, akin to the lymphatic vascular remodeling induced by filarial nematodes. Alternatively, the presence of M2 macrophages may alter the availability of host-derived nutrients or other molecules that the developing parasites require. Macrophage activation status is associated with profound changes in cell metabolism that could influence the concentrations of host factors in the immediate vicinity of larval schistosomes. Alternatively molecules associated with immunoregulation and type 2 responses may be recognized by schistosomes and utilized as signals of an environment that is appropriate for parasite development. A somewhat similar relationship was recently proposed to influence the development of *Litomosoides (L.) sigmodontis*, a filarial nematode, which speeds up its larval development and produces greater numbers of microfilaria in response to IL-5 and eosinophils. It was suggested that these parasites utilize IL-5 as a

predictor for future survival and altered life expectancy [65]. Studies to examine these and other possibilities in the context of schistosome infection are currently underway.

Here we presented evidence that regulation of pro-inflammatory processes is a contributor to determining the developmental fate of schistosomes in their definitive mammalian host. It remains to be determined how schistosomes might recognize a regulated immune environment and how this environment influences parasite development. However, these findings suggest that inflammation and its regulation are key components of a host environment permissive to schistosome infection and suggest that modulation of inflammatory processes may be a useful target for disrupting schistosome infection and could lead to new insights for improved treatments or vaccine development.

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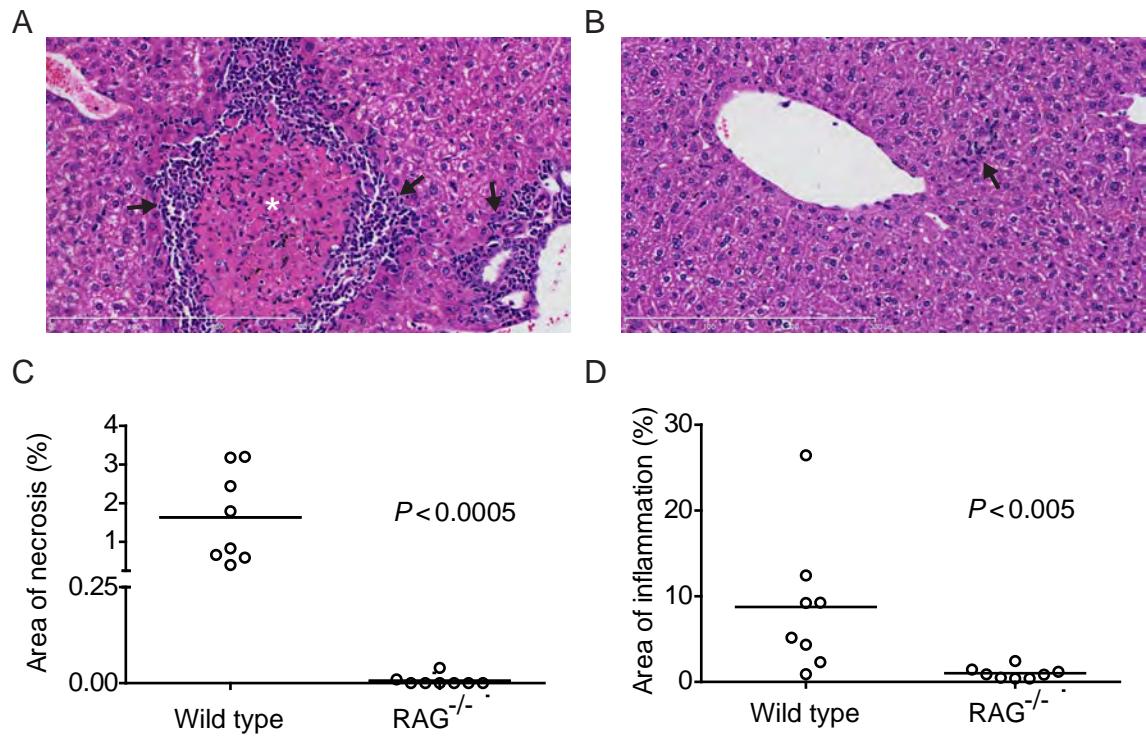
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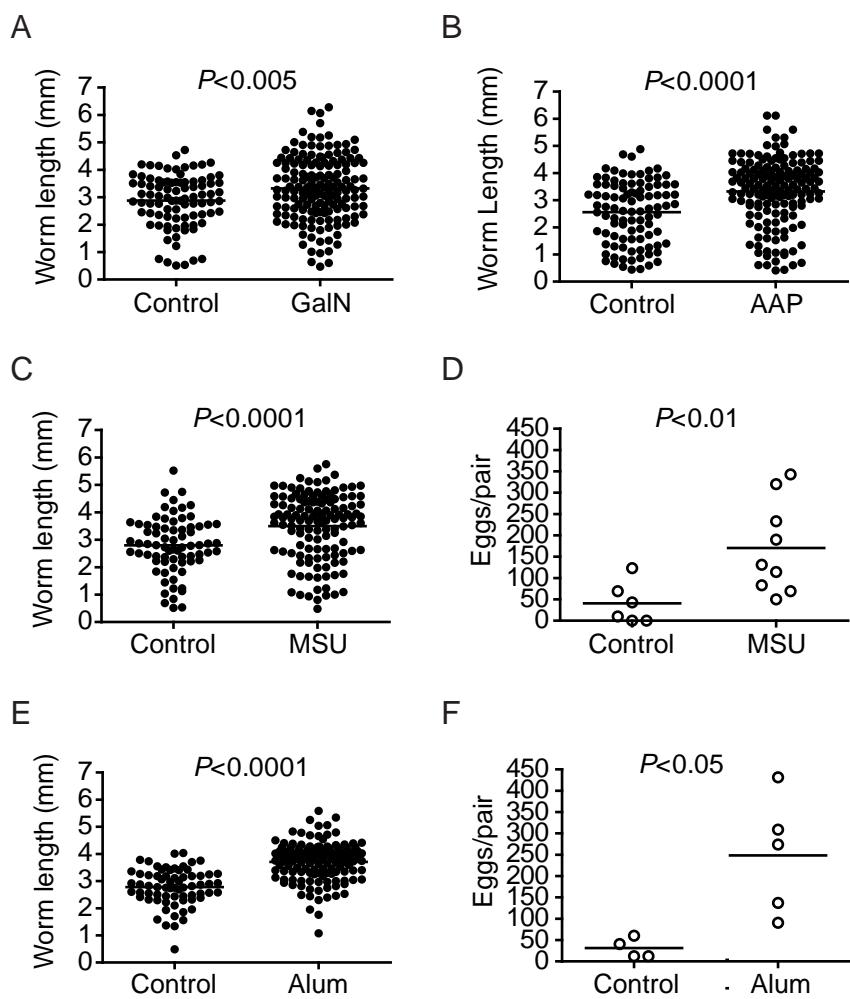
**Figure 1. Early *S. mansoni* infection fails to induce liver inflammation and coagulative necrosis in RAG<sup>-/-</sup> mice.**

Wild type (C57BL/6) and RAG<sup>-/-</sup> mice were infected with *S. mansoni* cercariae via percutaneous tail exposure. Digital images of liver sections (4 weeks p.i.) were obtained using Nanozoomer microscope and areas of inflammation and coagulative necrosis were measured using nanozoom digital software. (A) H&E stained liver section from a wild type mice exhibiting periportal infiltration of inflammatory cells, as well as, infiltration of inflammatory cells into the parenchyma (arrows). Example of coagulative necrosis also found in 4 wk infected wild type mice (\*). (B) H&E stained liver section from RAG<sup>-/-</sup> exhibiting only small areas of periportal infiltration of inflammatory cells (arrows). (C) Total percentage of coagulative necrosis was calculated per animal (n=8) and mean values are represented by horizontal bars. (D) Total percentage of inflammatory infiltrate was calculated per animal (n=8) and mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



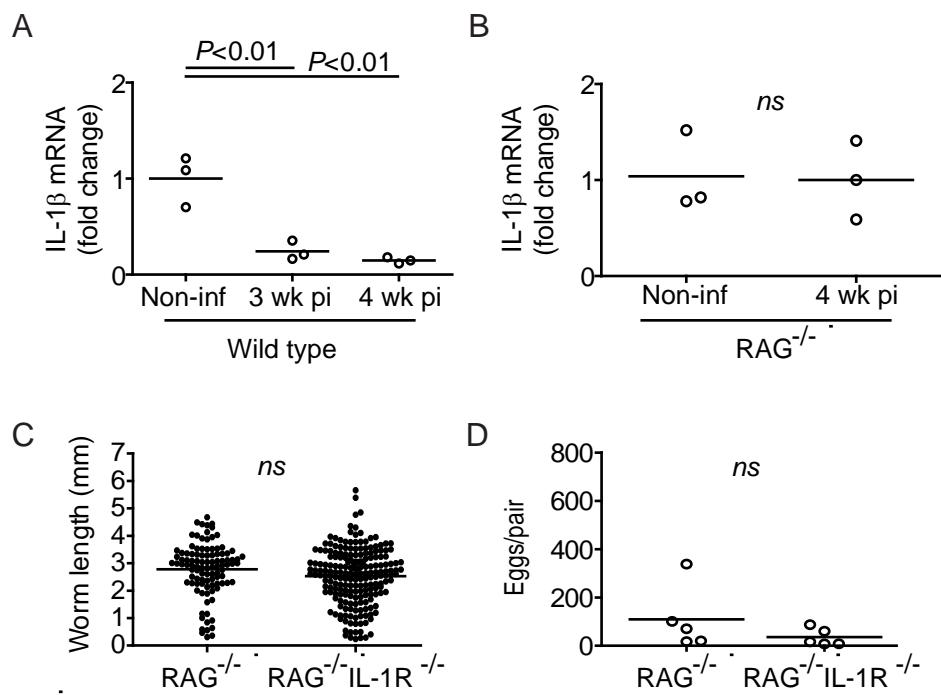
**Figure 2. Inducing hepatocyte death and inflammasome activation facilitates *S. mansoni* development in RAG<sup>-/-</sup> mice.**

Parasite development was restored after inducing chronic liver inflammation or chronic administration of NALP3 inflammasome agonists as determined by (A) Length of male worms recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with 10 mg D-(+)-galactosamine hydrochloride (GalN). (B) Length of male worms recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment once a week with 5 mg acetaminophen (AAP) for the first 3 weeks then 10 mg AAP for the last 3 weeks of infection. (C and E) Length of male worms and (D and F) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with 500 µg monosodium urate (MSU) or 1 mg alum. Mean values are represented by horizontal bars. Groups of 4 to 5 mice were used for each experimental condition. P values for worm lengths determined using student's T-test with Welch's correction. P values for egg production determined using the Mann Whitney test. Data shown for the MSU experiment is pooled from 2 independent experiments.



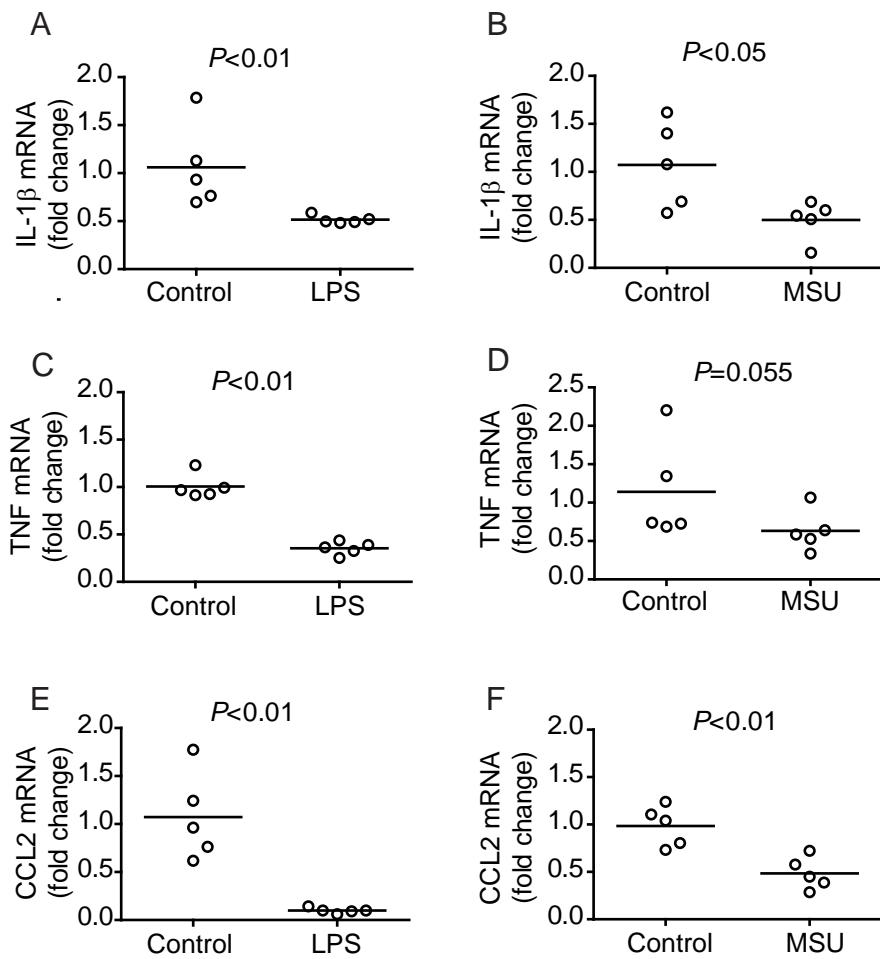
**Figure 3. *S. mansoni* development occurs in the context of down-regulated IL-1 $\beta$  expression.**

(A) Splenic IL-1 $\beta$  levels are down-regulated in 3 and 4 week schistosome infected C57BL/6 mice as compared to uninfected controls (B) Splenic IL-1 $\beta$  levels are unchanged in 4 week schistosome infected RAG $^{-/-}$  mice as compared to uninfected controls. Groups of 3 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined for 3 and 4 week infected wild type mice mRNA expression levels using an ANOVA with a Tukey post-test and for 4 week infected RAG $^{-/-}$  mice using the Mann Whitney test. (C) Length of male worms and (D) egg production by schistosome pairs recovered from RAG $^{-/-}$  or RAG $^{-/-}$  IL-1R $^{-/-}$  mice at 6 weeks p.i.. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test.



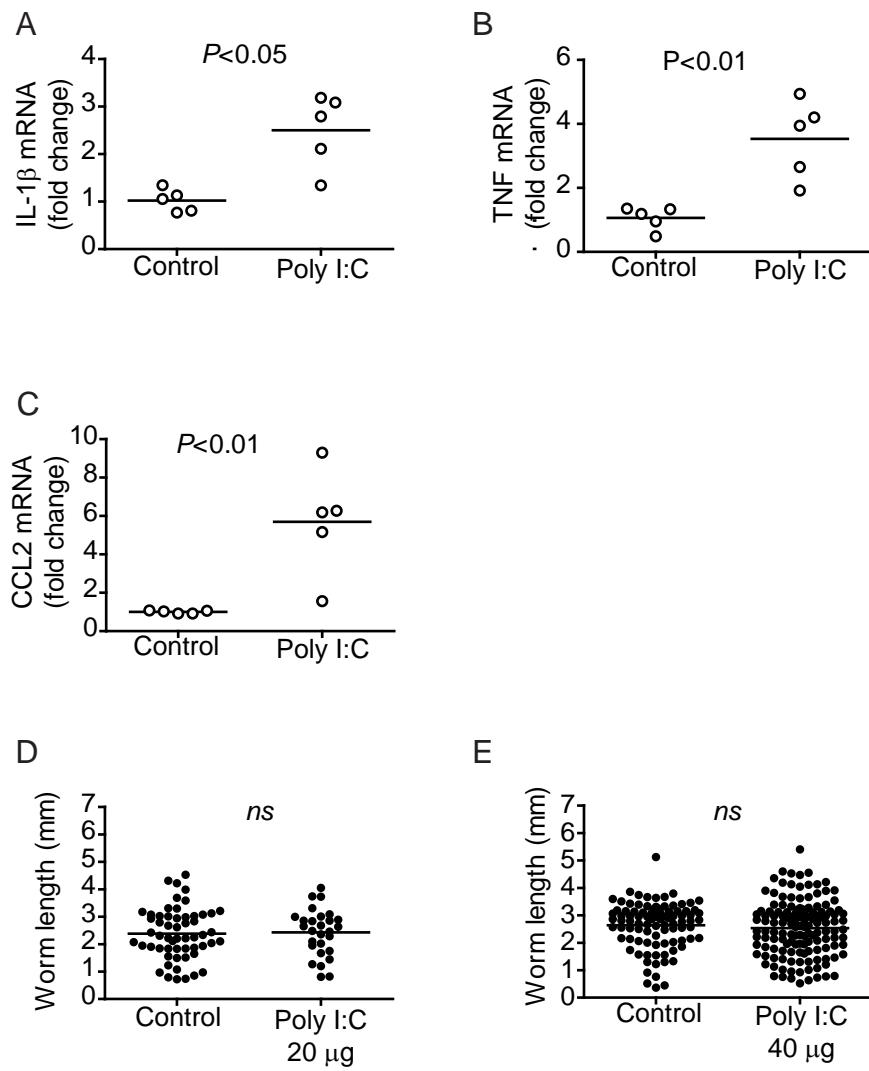
**Figure 4. Chronic TLR-4 or NALP3 inflammasome activation results in regulation of pro-inflammatory cytokine gene expression.**

Splenic IL-1 $\beta$  (A and B) TNF- $\alpha$  (C and D) and CCL2 (E and F) mRNA levels are down-regulated in 6 week schistosome infected RAG $^{-/-}$  mice treated twice a week with either 20  $\mu$ g LPS or 500  $\mu$ g MSU as compared to 6 week schistosome infected PBS treated RAG $^{-/-}$  mice. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



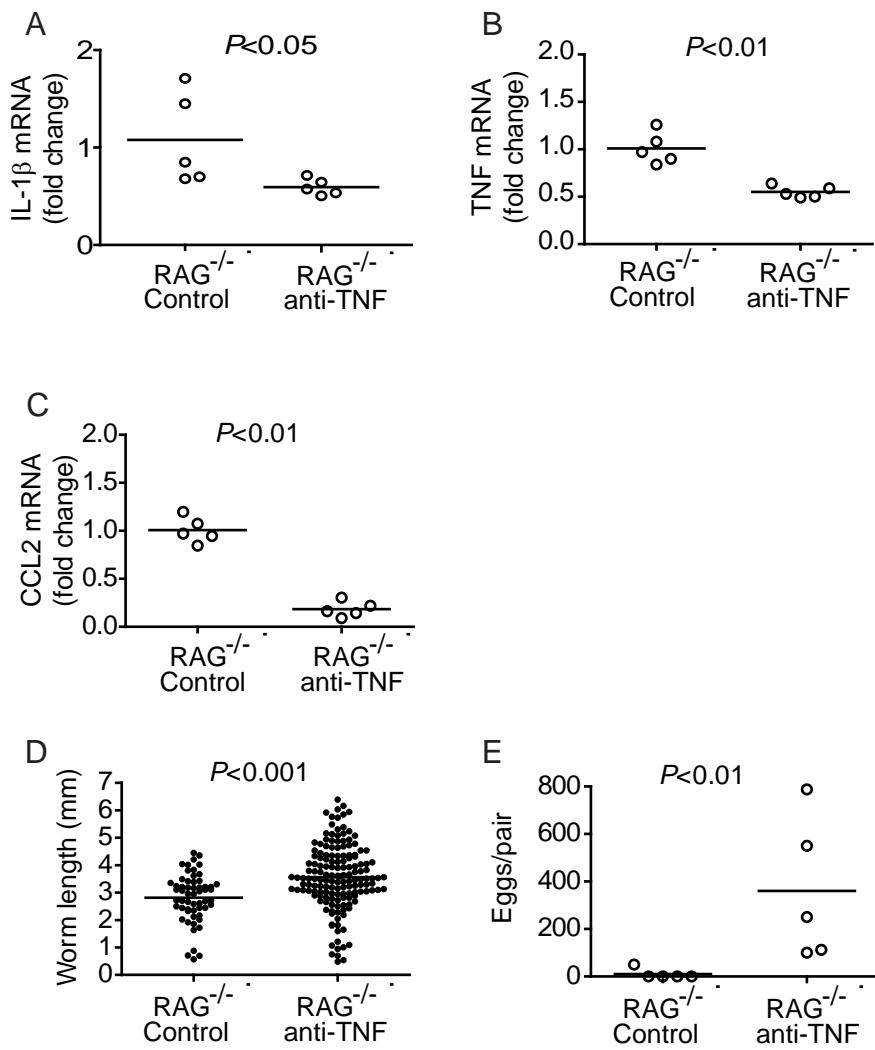
**Figure 5. Chronic TLR-3 signaling results in up-regulation of pro-inflammatory gene expression and fails to restore *S. mansoni* development in immunodeficient mice.**

Splenic (A) IL-1 $\beta$  (B) TNF- $\alpha$  and (C) CCL2 mRNA levels are up-regulated in 6 week schistosome infected RAG $^{-/-}$  mice treated twice a week with 20  $\mu$ g poly I:C. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test. (C) Length of male worms recovered from RAG $^{-/-}$  mice at 6 weeks p.i. after treatment twice a week with 20  $\mu$ g poly I:C. (D) Length of male worms recovered from RAG $^{-/-}$  mice at 6 weeks p.i. after treatment twice a week with 40  $\mu$ g poly I:C. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction.



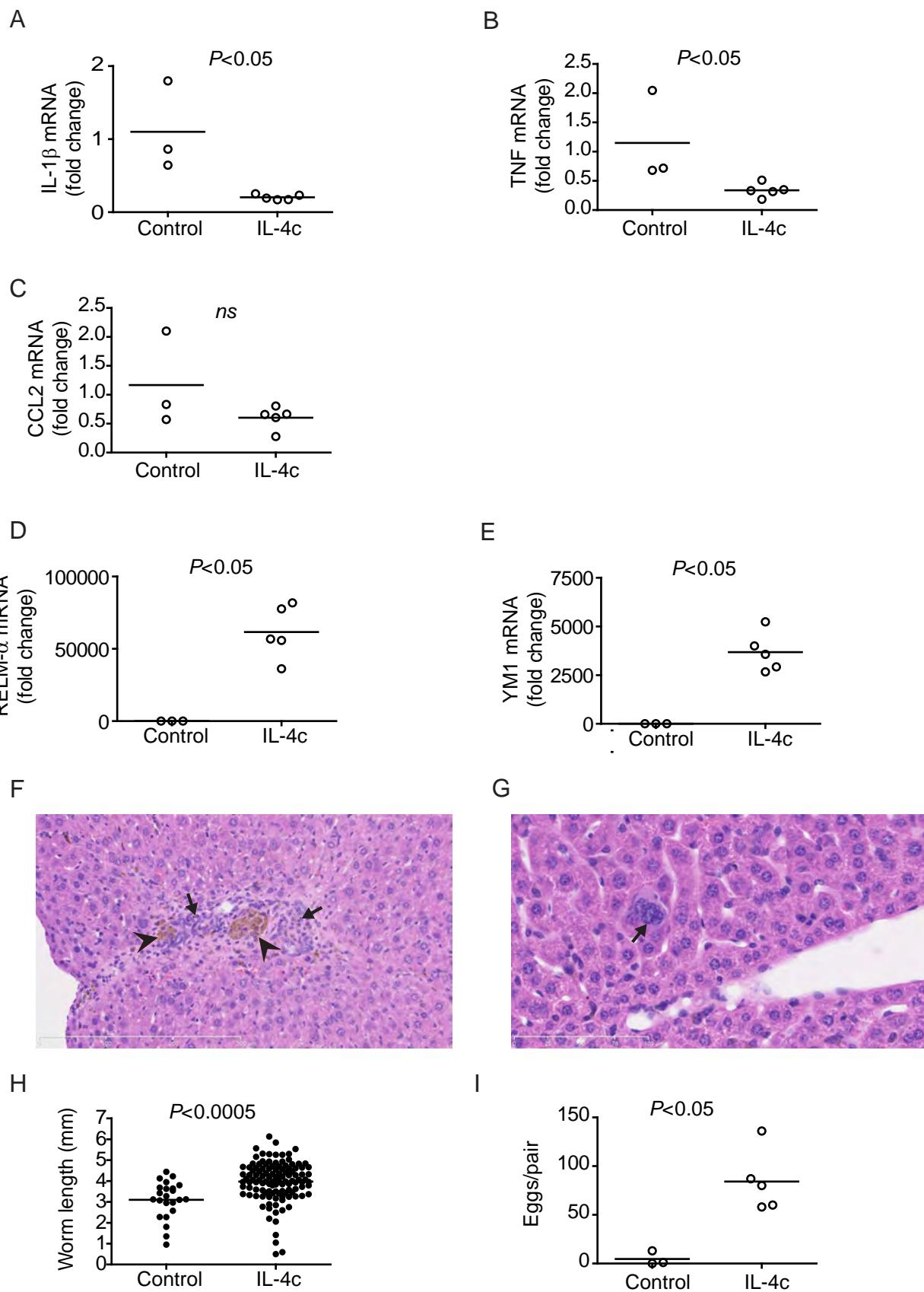
**Figure 6. Inducing regulation of pro-inflammatory cytokines via blockade of TNF restores *S. mansoni* development in RAG<sup>-/-</sup> mice.**

Splenic (A) IL-1 $\beta$  (B) TNF- $\alpha$  and (C) CCL2 mRNA levels are down-regulated in 6 week schistosome infected RAG<sup>-/-</sup> mice treated once a week with 100  $\mu$ g anti-TNF as compared to 6 week schistosome infected PBS treated RAG<sup>-/-</sup> mice. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test. Length of male worms (D) and (E) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment once a week with 100  $\mu$ g anti-TNF as compared to vehicle treated controls. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test.



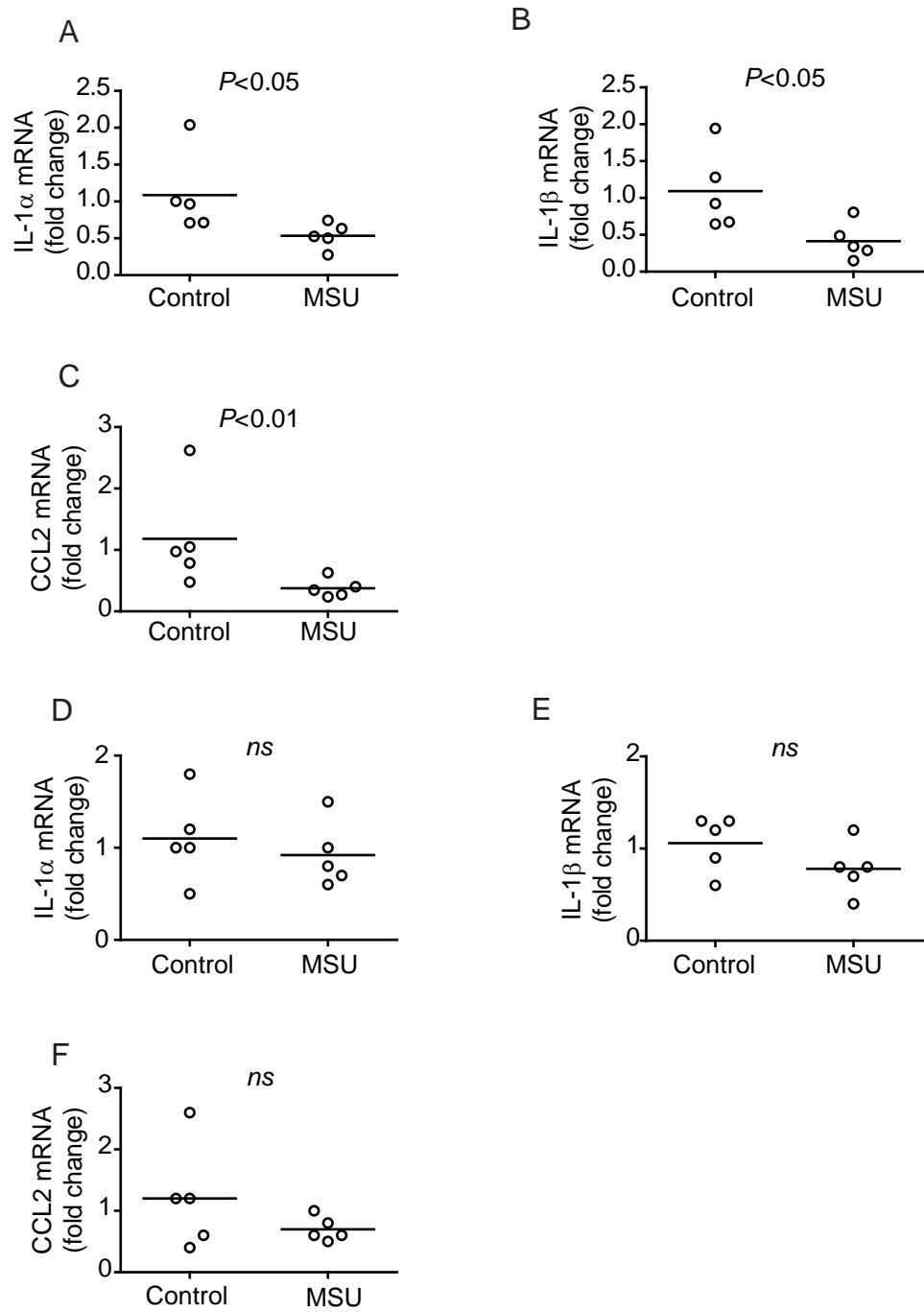
**Figure 7. IL-4 promotes a Th2-like environment that facilitates *S. mansoni* development in RAG<sup>-/-</sup> mice.**

Schistosome infected RAG<sup>-/-</sup> mice were treated weekly with 5 µg IL-4 complexed to 25 µg anti-IL-4 antibody 11B11 (IL-4c) for 6 weeks in order to promote a Th2-like environment. H&E images from a treated animal 6 weeks p.i. (A) highlighting macrophages (small arrows) in the inflammatory infiltrate surrounding worm pigment (large arrows) and (B) giant cell formation (arrows). ) Splenic (C) IL-1 $\beta$  and (D) TNF- $\alpha$  mRNA levels are down-regulated in IL-4c treated RAG<sup>-/-</sup> mice as compared to PBS treated RAG<sup>-/-</sup> mice. Splenic CCL2 (E) mRNA levels did not differ significantly between the experimental and control groups. P values were determined using the Mann Whitney test. Liver (F) RELM- $\alpha$  and (G) YM1 mRNA levels are upregulated in IL-4c treated RAG<sup>-/-</sup> mice as compared to PBS treated RAG<sup>-/-</sup> mice. Mean values are represented by horizontal bars. Groups of 3 to 5 mice were used for each experimental condition. (H) Length of male worms and (I) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice treated with IL-4c or PBS. Mean values are represented by horizontal bars. Groups of 3 to 5 mice were used for each group. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test.



**Supplemental Figure 1. Long term MSU administration results in the regulation of pro-inflammatory responses.**

Liver (A) IL-1 $\alpha$  (B) IL-1 $\beta$  and (C) CCL2 mRNA levels are down-regulated in 6 week schistosome infected RAG $^{-/-}$  mice treated twice weekly with 500  $\mu$ g MSU as compared to 6 week schistosome infected PBS treated RAG $^{-/-}$  mice. (D) IL-1 $\alpha$  (E) IL-1 $\beta$  and (F) CCL2 liver mRNA levels in RAG $^{-/-}$  mice return to baseline 18 hours after receiving a 500  $\mu$ g dose of MSU as compared to PBS treated controls. Mean values are represented by horizontal bars. Groups of 5 mice were used for each experimental condition. P values were determined using the Mann Whitney test.



## **Chapter 3**

**Restoration of *Schistosoma mansoni* development in immunodeficient mice by  
chronic TLR stimulation is dependent on MyD88 signaling**

## Abstract

Blood flukes of the genus *Schistosoma* infect 200 million people. As a result of host parasite co-evolution, *S. mansoni* has evolved to exploit host immune factors as signals to coordinate its own development within the host. Worms fail to develop normally in RAG<sup>-/-</sup> mice that lack all T and B cells, while development is restored when CD4<sup>+</sup> T cells are transferred into RAG<sup>-/-</sup> mice, suggesting that CD4<sup>+</sup> T cells play a central role in regulating parasite development. The role of CD4<sup>+</sup> T cells in this process is indirect, and is limited to the provision of non-cognate T cell help for innate responses which, in turn, facilitate parasite development. Indeed, T-cells can be bypassed completely in this process. The direct activation of innate immune system via chronic TLR stimulation via PAMPs in the absence of T-cells also restores worm development, indicating that innate immune signals are sufficient for parasite development to proceed normally. However, restoration of parasite development via chronic TLR stimulation requires MyD88 dependent signaling resulting in the regulation of pro-inflammatory cytokine transcription that creates an immune environment permissive for parasite development to proceed in immunodeficient mice.

## Introduction

Schistosomiasis, a disease caused by parasitic blood flukes, has plagued humans from antiquity [1] up to the present time, with an estimated 239 million people infected [2] and another approximately 700 million people at risk or acquiring the infection [3]. The vast majority of schistosome infections occur in sub-saharan Africa, with *S. mansoni* contributing significantly to this disease burden [4,5]. Schistosomiasis is responsible for an estimated annual loss of 70 million disability adjusted life years (DALYs), putting it ahead of malaria as parasitic cause for disease burden [6]. Once infected, an individual can harbor schistosomes for years, despite robust immune responses directed towards the parasite and its eggs.

As a result of extensive host-parasite co-evolution, *S. mansoni* and other schistosomes have likely evolved to exploit host immune factors to complete their development within the host, as parasite development is greatly attenuated in immunodeficient hosts. Host CD4<sup>+</sup> T cells appear central to parasite development, as CD4<sup>+</sup> T cells are sufficient to restore parasite development in immunodeficient hosts [7,8]. However, the role that T cells play in this process is indirect and is mediated through provision of T cell help for innate immune components, as the requirement for CD4<sup>+</sup> T cells can be circumvented completely by the direct stimulation of innate immune responses via lipopolysaccharide- (LPS-) mediated activation of toll-like receptor(TLR)-4 [9].

While there are numerous examples of bacterial, viral, fungal, and protozoan pathogen associated molecular patterns (PAMPs) [10-12] worm antigens have not been shown to robustly activate innate immune responses via TLR signaling. Furthermore, while LPS has been shown to reach significant concentrations in the blood of schistosomiasis patients, this is probably the result of intestinal mucosal damage induced by parasite eggs and is unlikely to occur before the onset of oviposition, when the parasites are developing. These two observations brought into question the physiological relevance of TLR4 activation in schistosome development.

We therefore sought to establish whether *S.mansoni* development in RAG<sup>-/-</sup> mice could be restored through chronic stimulation with other bacterial, viral and fungal PAMPs that signal through other TLRs. As TLRs can be broadly categorized based on subcellular localization as either plasma membrane-bound or endosomal, we also tested whether receptor location impacted any potential effects on parasite development. Finally, as TLR signaling is propagated by two distinct pathways, distinguished by involvement of two different adaptor proteins, MyD88 and TRIF, we tested whether there was evidence for preferential involvement of either pathway in stimulating parasite development.

## Materials and Methods

Ethics statement:

All animal studies were conducted in accordance with established protocols approved by the USUHS Institutional Animal Care and Use Committee.

Experimental mice:

RAG-1<sup>-/-</sup> mice on a C57BL/6 background were originally purchased from Jackson laboratory (Bar Harbor, ME) and then bred in-house for experimental use. All mice used in experiments were age matched.

Parasite parameters:

Mice were infected percutaneously via tail exposure to water containing 160 *S. mansoni* cercariae (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails. At 6 weeks post infection (p.i.) mice were sacrificed. Worms were perfused from the portal system and immediately fixed in 4% neutral buffered formaldehyde. Male and female worms were counted and photographed at 20x magnification using a Nikon D80 10.0 megapixel digital camera attached to a Zeiss trinocular dissecting microscope. Worm growth was assessed by measuring the length of male worms from digital micrographs Image J software (<http://rsb.info.nih.gov/ij>), as described previously[9]. Only

male worms were measured as female growth is dependent upon receiving developmental cues from pairing with maturing males [13]. Sexual maturation of the parasites was assessed by calculating egg production per worm pair from liver egg burdens, as described previously [9]. Briefly, liver tissue was homogenized and digested in 0.7% trypsin phosphate buffered saline (PBS) solution and the released eggs were counted under a dissecting microscope.

#### TLR stimulation:

RAG-1<sup>-/-</sup> mice were infected with cercariae as described above. Mice received biweekly i.p. 20 µg/mouse injections of one of the following Invivogen TLR ligands: Pam3CSK4 or zymosan (TLR2), poly I:C (TLR3), ultrapure LPS *E.coli* 0111:B4 or MPLA (TLR4), Guardiquimod (TLR7), CpG (TLR9). Control mice received biweekly i.p. injections of PBS without calcium and magnesium. At 6 weeks p.i. mice were sacrificed and parasite parameters were determined as described above.

#### RNA isolation, purification, and Real time PCR

RNA was isolated from the spleens and/or livers of RAG-1<sup>-/-</sup> mice. After removal, tissues were immediately placed in 1 ml RNA-BEE (Tel-Test, Friendswood, Texas), homogenized, snap-frozen in liquid nitrogen and stored at -80°C until isolation of total RNA, following manufacturer's instructions. RNA was further purified following the RNeasy mini protocol for RNA cleanup (Qiagen, Valencia, California). Purified

RNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). 2 ug of RNA was used for cDNA preparation using a high capacity RNA to cDNA kit (Invitrogen, Grand Island, New York) following manufacturer's instructions. Real time PCR was performed with a MJ Research Chromo4 PTC-200 thermocycler unit (Bio-Rad, Hercules, CA) using Taqman gene expression assays and TaqMan gene expression master mix (Invitrogen) following manufacturer's instructions. Assays for the following mRNAs were performed: *rsp29*, GAPDH, IL-1 $\beta$ , TNF- $\alpha$ , CCL2, IFN- $\gamma$ , IL-6, IL-10, and NOS2. Expression of genes of interest was normalized to the expression of GAPDH or *rsp29* and fold changes in expression were calculated following the  $2^{-\Delta\Delta CT}$  method [14].

#### Statistical analysis

All statistical analyses were performed using GraphPad Prism Inc. version 4.0 software (San Diego, California). Significant differences for worm lengths between two groups were determined using a student's unpaired T-test with Welch's correction. Significant differences for worm lengths between 3 or more groups were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. The Mann-Whitney test was utilized to determine significant differences for both eggs/worm pairs as well as splenic or liver fold changes between two groups. *P* values of less than 0.05 were considered significant. 3-5 mice were used per experimental group and each experiment was repeated at least twice.

## Results

### *TLR1/2 stimulation by PAM3CSK4 restores parasite development in immunodeficient mice*

As innate immune activation via LPS and TLR4 signaling has been shown to restore parasite development in RAG<sup>-/-</sup> mice, we tested whether other TLR ligands that shared similar signaling pathways to TLR4 could also restore parasite development in these animals. TLR1/2 and TLR2/6, like TLR4, utilize the adaptor proteins MYD88 and TIRAP/MAL for signaling [10]. We found that administration of Pam3CSK4, a TLR1/2 agonist [15], during prepatent infection resulted in partial restoration of parasite development, as evidenced by increased parasite size (Figure 8A), but egg production, while trending upward, did not differ significantly from the vehicle control group (Figure 1B). On the other hand, administration of zymosan, a yeast TLR2/6 agonist [16], during prepatent infection failed to restore parasite size (Figure 8A) or result in parasite egg production (Figure 8B). Previously, we have shown that restoration of parasite development by LPS coincides with the downregulation of splenic IL-1 $\beta$ , TNF, and CCL2 gene transcription (Chapter 2 Figure 4A-D). As chronic administration of PAM3CSK4 restored parasite size, we hypothesized that this treatment would result in a similar pattern of transcriptional changes. Instead, we found that at 6 weeks post infection, IL-1 $\beta$ , TNF- $\alpha$ , and CCL2 splenic mRNA levels were unchanged in PAM3CSK4-treated animals compared to vehicle controls (Figure 8C-E). However, both IFN- $\gamma$  and IL-6 (Figure 8F-G) mRNA levels were downregulated in the spleens of

PAM3CSK4-treated animals. In contrast, we found that splenic IL-10 mRNA levels were significantly upregulated in PAM3CSK4-treated mice, being 5- to 40-fold higher compared to controls (data points included in Figure 8H). In zymosan-treated animals, there were no significant changes in inflammatory cytokine or IL-10 mRNA (data not shown). These data support our previous conclusion that schistosome development occurs in the context of regulated pro-inflammatory responses and suggests that IL-10 plays a role in regulating inflammation induced by chronic PAM3CSK4 administration.

*Restoration of parasite development in immunodeficient mice is specifically associated with MyD88-dependent regulation and not TLR location*

TLR4 signaling is unique in that it utilizes both the ubiquitous MyD88 adaptor protein and TRIF, a second adaptor protein that is shared only with TLR3 [17]. TLR3 signaling is unique in that it signals solely through the adaptor TRIF [18]. Previously we showed that chronic administration of poly I:C, a mimic of viral double-stranded RNA and a TLR3 agonist [19], failed to restore parasite development in RAG<sup>-/-</sup> mice (Chapter 2 Figure 5D-E)). We therefore hypothesized that parasite development is specifically linked to chronic MyD88-dependent stimulation and does not occur in response to TRIF stimulation. To test this hypothesis, we tested whether TRIF-specific signaling via TLR4 would restore parasite development in immunodeficient mice. We found that when we chronically treated infected RAG<sup>-/-</sup> mice with monophosphoryl lipid A (MPLA), a TRIF-selective TLR-4 agonist [20], during pre-patent infection, parasite development was not restored. Worms recovered from treated animals did not differ significantly in size

(Figure 9A) or in egg output (data not shown) when compared to vehicle controls. These data suggest that TRIF-mediated signaling alone is not conducive to parasite development and that restoration of parasite development via LPS stimulation of TLR-4 occurs specifically via MyD88 signaling. An alternative explanation we considered for why poly I:C failed to restore parasite development was that, unlike TLR4 and TLR1/2, which are expressed on the plasma membrane, TLR3 is located intracellularly on endosomal membranes. To determine whether TLR location played a role in schistosome development, we tested whether ligands for TLR-7 and TLR-9, which are both endosomal TLRs, could restore parasite development in RAG<sup>-/-</sup> mice. We found that chronic administration of CpG, a TLR-9-activating synthetic oligodeoxynucleotide sequence containing unmethylated CG dinucleotides [21,22] partially restored parasite growth compared to controls that received vehicle alone (figure 9B), while egg production by the worms was unchanged (data not shown). Chronic administration of gariquimod, a TLR-7-activating imidazoquinoline compound [23], also restored parasite growth (Figure 9C), while egg production trended upwards but narrowly avoided attaining statistical significance (Figure 9D). These findings suggested that TLR location is unimportant with regard to stimulating schistosome development and that the failure of TLR3 signaling to influence parasite development is not due to its endosomal location. Furthermore, these findings suggest that parasite development can be restored by MyD88-dependent TLR stimulation and that the failure of poly I:C (and MPLA) to restore parasite development is due to their utilization of TRIF-dependent signaling.

We further examined the inflammatory cytokine profile resulting from chronic gariquimod treatment as TLR7, like TLR3, is important for recognition of viral PAMPs,

and we had previously shown that chronic administration of poly I:C resulted in a sustained pro-inflammatory response rather than down-regulation. We found that chronic stimulation with gardiquimod resulted in the downregulation of both TNF and NOS2 (Figure 9E-F) splenic mRNA levels, While splenic mRNA levels for IL-1 $\beta$ , CCL2, IFN- $\gamma$ , IL-6 and IL-10 did not differ from controls (Figure 9G-K). Thus, chronic TLR7 stimulation induced a transcriptional profile distinct from that of chronic TLR3 stimulation, with evidence of down-regulation of inflammatory signals rather than up-regulation. These data suggest that failure of parasites to develop after chronic poly I:C stimulation is linked to the sustained up-regulation of pro-inflammatory cytokine transcription that is unique to poly I:C and TLR3 signaling.

*Transcriptional changes associated with restoration or failure of parasite development*

In light of the inflammatory cytokine mRNA transcription patterns generated in response to chronic TLR1/2 and TLR7 stimulation, we tested whether any of these transcriptional changes were also associated with treatments we had previously identified as either restorative (LPS) or not restorative (poly I:C) of parasite development. Like PAM3CSK4 (TLR1/2) stimulation, chronic LPS stimulation also resulted in the downregulation of splenic IL-6 and IFN- $\gamma$  (Figure 10A-B) and, like gardiquimod (TLR7), the downregulation of NOS2 (Figure 10C). However, unlike PAM3CSK4, chronic LPS stimulation did not result in the upregulation of IL-10 and instead, IL-10 transcription levels did not differ from the control group (Figure 10D). This finding suggests that IL-10 may play a more significant role in the regulation induced by chronic

administration of PAM3CSK4 than by chronic administration of LPS. In contrast, chronic poly I:C stimulation resulted in upregulation of IFN- $\gamma$  and NOS2 (Figure 10F,-10G), showing again that chronic poly I:C administration results in sustained inflammation. Interestingly, chronic poly I:C administration also induced upregulation of IL-10 transcription (Figure 10H), in conjunction with inflammatory genes, indicating that this regulatory cytokine is induced by poly I:C, despite the overall pattern of sustained inflammation associated with poly I:C administration.

*Lack of correlation between presence of parasite eggs and liver cytokine transcription*

Our data demonstrate that restoration of parasite development in RAG<sup>-/-</sup> mice occurs in the context of systemic downregulation of pro-inflammatory responses, as evidenced by the reduced transcription of inflammatory genes in the spleen that correlates with restored parasite development. However, during *S. mansoni* development from 1-2 weeks post infection until adulthood occurs in portal venules of the liver, suggesting the liver may be a more relevant site of inflammation and inflammatory regulation early in infection. Indeed, in wild type mice, obvious histological changes of inflammation can be observed during pre-patent infection, in association with areas of hepatocellular necrosis. However analysis of gene expression in the liver is complicated by the fact that, in situations where parasite development is restored, parasite eggs will also begin to accumulate in the liver after week 5 of infection, possibly influencing transcription patterns further. To gain insights into how liver gene expression correlates with parasite development and egg deposition, we examined liver transcription of TNF and IL-10 following treatments that enhance parasite development either with (LPS) or without

(gardiquimod, PAM3CSK4) inducing significant parasite egg accumulation, and compared them with poly I:C treatment, which does not restore parasite development. We found that in animals where treatment fully restored parasite development (LPS), hepatic TNF and IL-10 transcription was modestly elevated relative to control animals (Figure 11A, 11B). However, TNF and IL-10 transcript levels in livers of PAM3CSK4- and gardiquimod-treated animals, where significant egg accumulation did not occur, were either unaffected by treatment (gardiquimod, Figure 11C, 11D) or were strongly up-regulated (PAM3CSK4, Figures 11E, 11F). In the case of the latter, PAM3CSK4 treatment induced an almost 10-fold increase in TNF transcription (Figure 11E), which was accompanied by increase in IL-10 transcription of over 20-fold (Figure 11F). Thus there was no discernible pattern of expression that correlated with the accumulation of parasite eggs.

In comparison, poly I:C treatment also induced an almost 10-fold increase in TNF mRNA in the liver (Figure 11H), but while IL-10 transcription was also significantly increased by poly I:C (Figure 11I), this increase was more modest, on the order of a two-fold increase relative to controls. These findings were in contrast to those obtained with PAM3CSK4. While both stimuli induced robust TNF transcription, only PAM3CSK4 also induced IL-10 at levels that exhibited a strong positive correlation with the induction of TNF (Figure 11G,  $R^2 = 0.9, P < 0.05$ ). Poly I:C induced both TNF and IL-10, but the induction of the latter did not correlate with the former (Figure 11J). Thus poly I:C, which does not restore parasite development, again induced an immunological milieu where induction of inflammatory signals was not accompanied by regulation. Taken together this data suggests regulation of pro-inflammatory responses occur in the spleen

rather than the liver. It also once again highlights poly I:C's remarkable ability to maintain sustained inflammation in both the spleen and the liver.

## Discussion

Innate immune responses to acute PAMP/TLR stimulation have been extensively studied and described in the literature [10,11,24,25]. The phenomenon of endotoxin tolerance, where administration of small doses of LPS induces a state of tolerance to subsequent lethal high dose exposure, has also been well characterized [26,27]. However, to our knowledge there are no reports in the literature describing and comparing innate immune responses induced by chronic exposure to various TLR ligands. Here we show that chronic TLR stimulation results in profoundly different immune states, depending upon the PAMP/TLR combination used, and that these different immunological milieus have different effects on the development of *S. mansoni* in immunodeficient mice.

Administration of the various TLR ligands to RAG<sup>-/-</sup> mice resulted in three different outcomes. The first, observed after chronic administration of LPS, Pam3CSK4 or gardiquimod, resulted in the regulation of pro-inflammatory cytokine mRNA transcription and was associated with parasite development. Second, chronic administration of the ligands MPLA and zymosan resulted in little change in cytokine mRNA levels when compared to controls and did not restore parasite development. Finally, chronic administration of Poly:Ic resulted in up-regulated pro-inflammatory cytokine mRNA levels, but also did not restore parasite development in RAG<sup>-/-</sup> mice. These results suggest that schistosome development requires the initiation of inflammation and the subsequent induction of regulatory mechanisms to control that inflammation, two criteria that are only met in these experiments when infected animals are administered, LPS, PAM3CSK4 or gardiquimod.

Unlike ligands for TLR-2, TLR-4, and TLR-9 [28-31], we found that repeated administration of Poly:Ic did not induce tolerance to further administration of this stimulus. Instead, chronic administration of Poly-Ic resulted in robust and sustained pro-inflammatory responses (Figure 10E-G and Figure 11H and 11I) and appears to be unique to among the TLR ligands we have examined. Unlike MyD88-dependent ligands, which promote tolerance to one another, TRIF dependent signaling fails to induce tolerance to MyD88-dependent PAMPs and pre-treatment with Poly-Ic, followed by treatment with a MyD88-dependent ligand, resulted in synergistic pro-inflammatory responses [32].

The specific PAMP administered also had an impact on the immune state generated after chronic TLR stimulation. Both zymosan and PAM3CSK4 are TLR-2 ligands, but chronic administration of these ligands results in quite different results. Acute administration of zymosan has been shown to induce IL-10 production, but little to no IL-6 or IL-12(p70), and has been characterized as inducing immunological tolerance [33]. In contrast, acute PAM3CSK4 administration results in robust production of not only IL-10, but also IL-6 and IL-12(p70) [34], as well as TNF [35]. Chronic administration of PAM3CSK4 resulted in the regulation of these pro-inflammatory responses and restored *S. mansoni* development, whereas chronic administration of zymosan had no significant effects on splenic pro-inflammatory cytokine or IL-10 mRNA levels and did not restore parasite development. While the PAM3CSK4 results demonstrate that TLR2 signaling can restore parasite development, we speculate that zymosan failed in this regard because of its failure to induce significant inflammation or regulation after chronic administration.

An alternative explanation for the failure of zymosan and MPLA to create a permissive environment for *S. mansoni* development involves the dosage utilized. Our dosage of 20 µg twice a week is similar to the dosage used to acutely induce immunological tolerance [33], whereas higher doses involving milligrams have been used to induce autoimmune arthritis [36]. Likewise, MPLA has been reported to have only 0.1% of the inflammatory toxicity as LPS, the parent molecule from which it is derived [20,37,38], so while in our hands, 20 µg of LPS was an adequate dose to stimulate inflammation and subsequent regulation, the same dose for MPLA may not have been sufficient for this to occur. However, it is questionable that increasing the dosage of MPLA would result in significantly higher production of pro-inflammatory cytokines. Unlike TRIF dependent poly I:C/TLR3 stimulation, which results in the production of type 1 interferons [39] and pro-inflammatory cytokines such as TNF and IL-6 [32] TRIF-dependent MPLA signaling through TLR4 only resulted in production of IFN- $\beta$  [20].

The manipulation of innate immune responses by chronic TLR signaling has provided insights into the types of innate responses that support parasite development and those that do not. From these studies, we conclude that regulation of pro-inflammatory responses by chronic TLR agonist administration favors parasite development. We previously showed that administration of IL-4, the hallmark cytokine of Th2 responses, also restores parasite development in RAG<sup>-/-</sup> mice. From that finding and the results presented here, we propose that immunoregulation supportive of parasite growth can occur via different mechanisms. In our experimental RAG<sup>-/-</sup> system, chronic administration of an appropriate TLR agonist can result in regulation of pro-inflammatory signals that promotes parasite growth. However, in immunologically intact

hosts, we propose that Th2 responses provide the regulation of pro-inflammatory signals that is necessary for schistosome development to proceed. A clearer understanding of immune factors that favor parasite development may be relevant to the development of vaccines that prevent schistosome infection [40-42]. To date the most protective vaccine in animal models is the irradiated cercarial vaccine [40,43,44] that induces partial immunity mediated by Th1 responses [45-47]. In contrast, alum, a vaccine adjuvant known to promote Th2-biased responses [48,49], abrogated the protection induced by a DNA vaccine based on a 23 k-Da *S. mansoni* integral membrane protein, a vaccine that, when used alone, induced up to 44% protection to challenge infection [50]. These observations suggest that Th1, rather than Th2 responses may be more successful in mediating protection against schistosome infection. However, there are data to suggest that Th2 responses also participate in mediating protection after vaccination [51,52], as the responses to secondary challenge with normal cercariae after irradiated cercarial vaccination are Th2 and IL-4 mediated [53]. Our data suggest that regulation of pro-inflammatory responses, by Th2 responses or by TLR/Myd88-mediated tolerance, appear to favor parasite development, indicating that maintenance of pro-inflammatory responses to schistosomes may be more effective in disrupting infection.

The data we present here provide insights into how schistosome infection may be influenced by responses induced by TLR agonists, some of which have been proposed to be useful as vaccine adjuvants [54-58]. It remains to be elucidated how schistosomes recognize a regulated immune environment and how that environment is beneficial to the parasite. However, our data suggest that augmentation of inflammatory processes could

be useful for disruption of schistosome infections and to inducing protection from schistosome mediated pathology.



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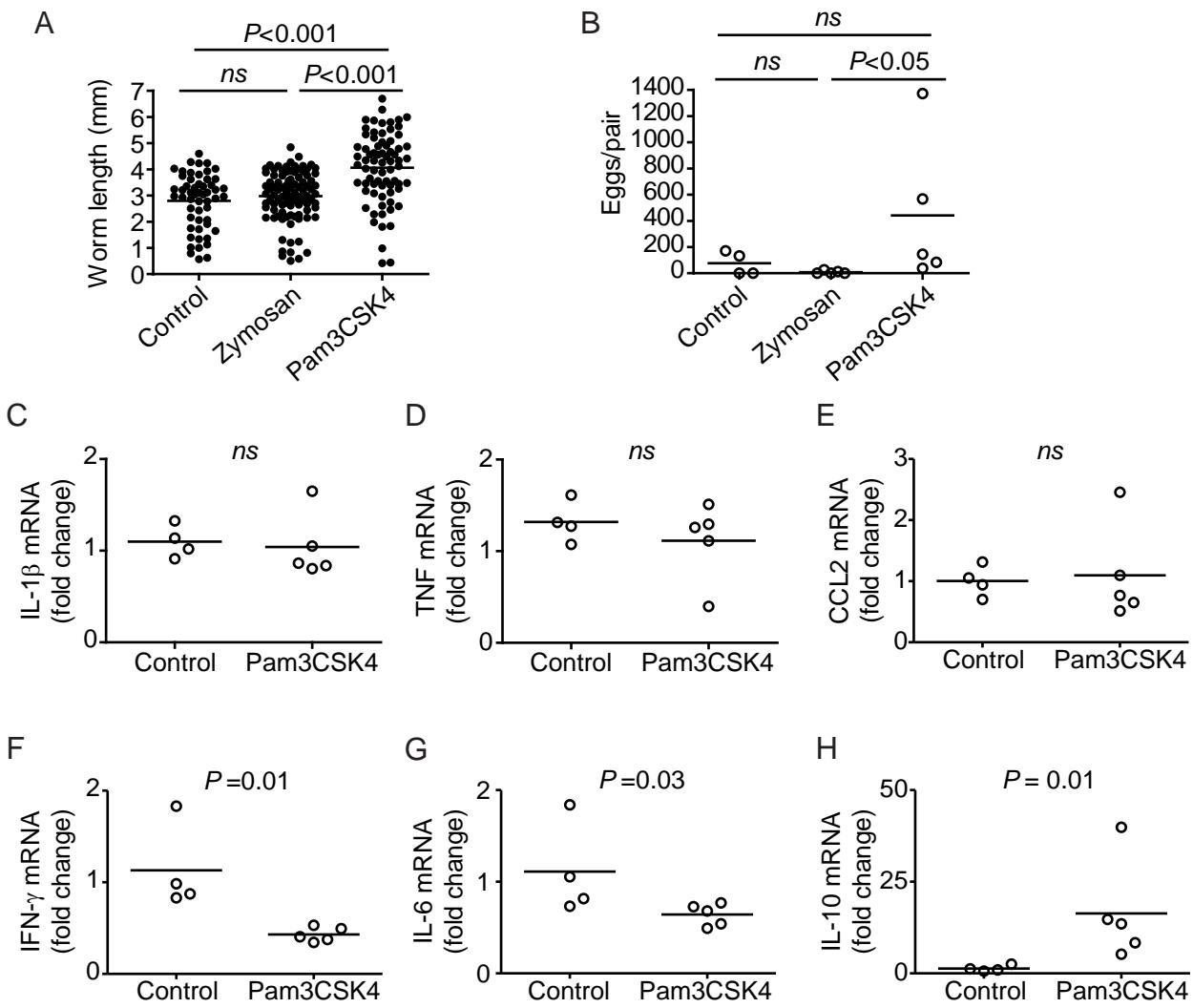
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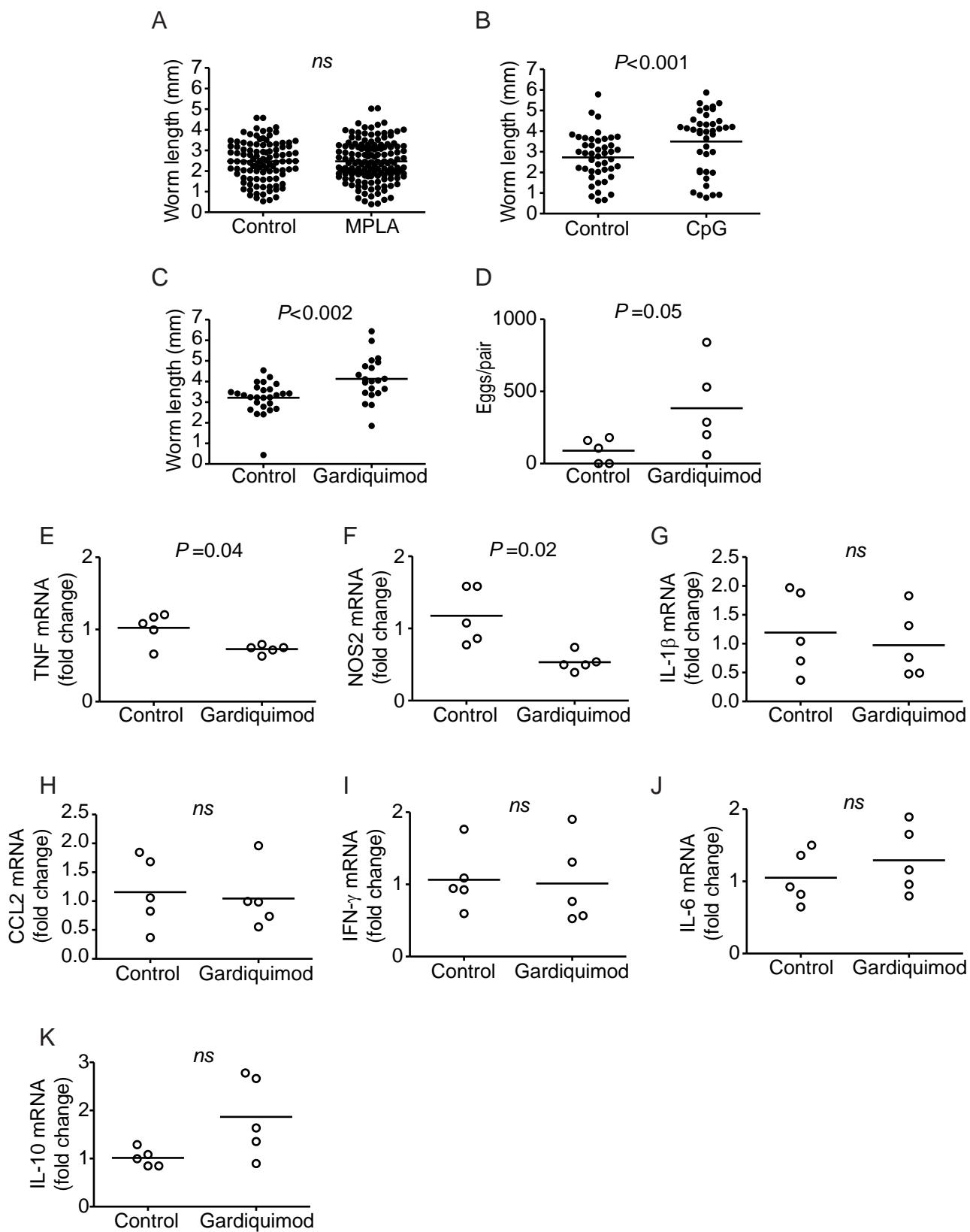
**Figure 8. Chronic stimulation of TLR2 with bacterial Pam3CSK4 facilitates *S. mansoni* development in RAG<sup>-/-</sup> mice and results in regulation of pro-inflammatory cytokine gene expression.**

(A) Length of male worms or (B) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with either vehicle control, 20 µg Pam3CSK4, or 20 µg zymosan. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test. Splenic mRNA levels for (C) IL-1 $\beta$  (D) TNF- $\alpha$  and (E) CCL2 were unchanged while splenic mRNA levels for (F) IFN- $\gamma$  and (G) IL-6 were down-regulated and (H) splenic IL-10 levels were up-regulated in 6 week schistosome infected RAG<sup>-/-</sup> mice treated twice a week with 20 µg Pam2CSK4. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



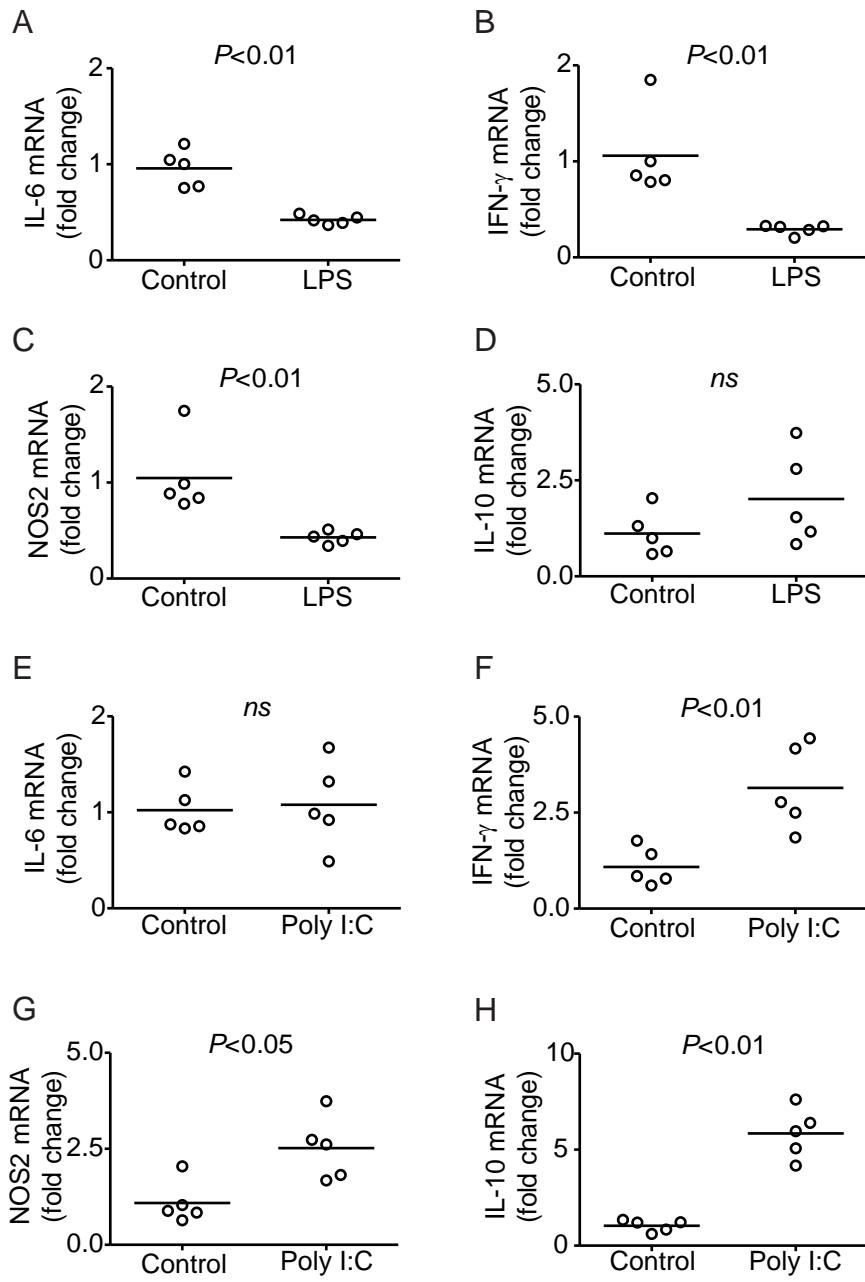
**Figure 9. Restoration of parasite development in RAG<sup>-/-</sup> mice after chronic TLR stimulation is MyD88 dependent.**

(A) Length of male worms recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with either vehicle control or 20 µg MPLA. (B) Length of male worms recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with either vehicle control or 20 µg CpG. (C) Length of male worms or (D) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with either vehicle control or 20 µg guardiquimod. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test. Splenic mRNA levels for (E) TNF- $\alpha$  and (F) NOS2 were down-regulated while splenic (G) IL-1 $\beta$  (H) CCL2 (I) IFN- $\gamma$  (J) IL-6 and (K) IL-10 mRNA levels were unchanged in 6 week schistosome infected RAG<sup>-/-</sup> mice treated twice a week with 20 µg guardiquimod. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



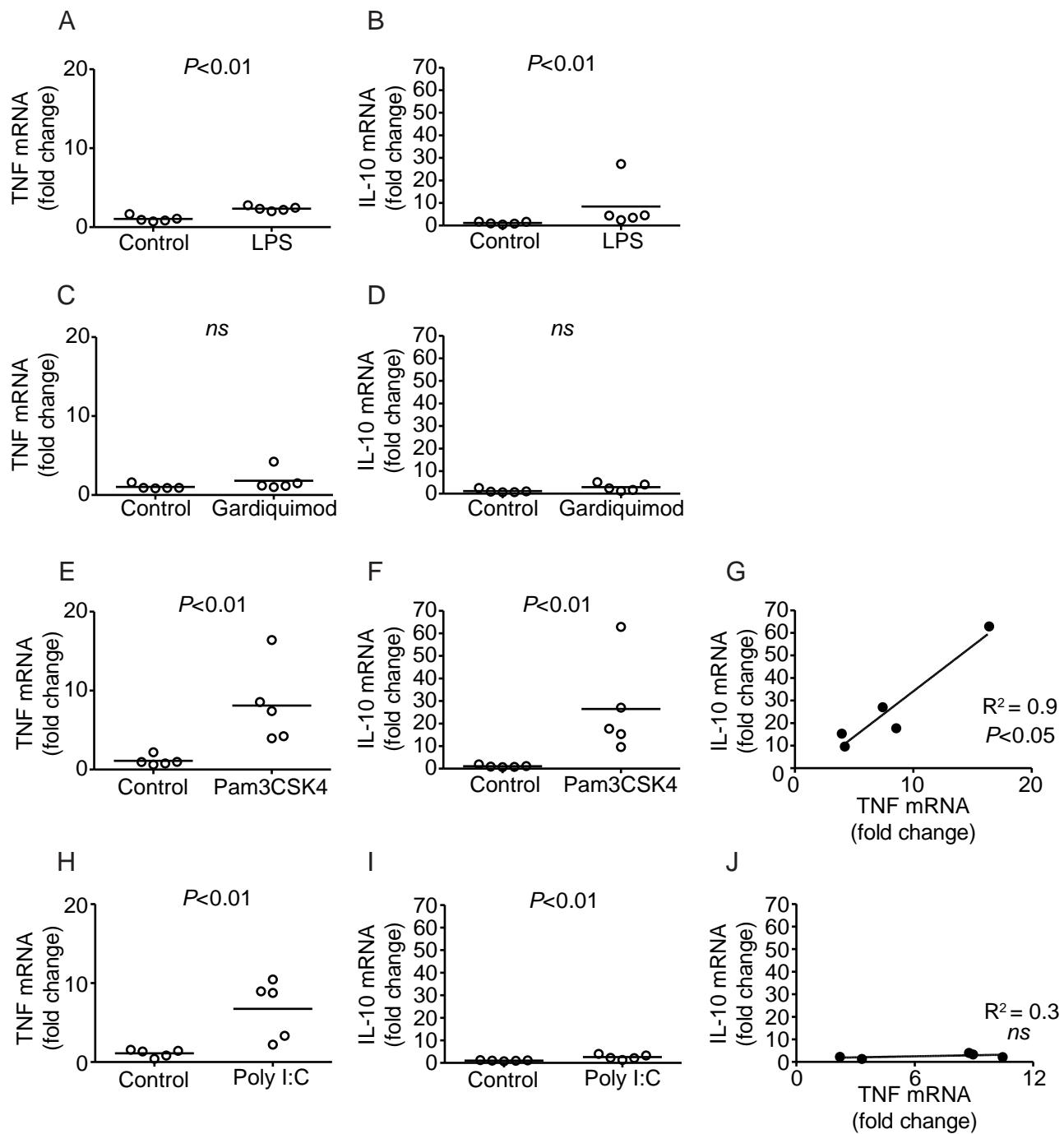
**Figure 10. Chronic TLR-4 signaling via LPS results in downregulated pro-inflammatory gene expression while chronic TLR-3 signaling via poly I:C results in the up-regulation of pro-inflammatory gene expression.**

Splenic mRNA levels for (A) IL-6, (B) IFN- $\gamma$ , and (C) NOS 2 were down-regulated while splenic mRNA expression levels were unchanged for (D) IL-10 in 6 week schistosome infected RAG<sup>-/-</sup> mice treated twice a week with 20  $\mu$ g LPS. Splenic mRNA levels for (E) IL-6 were unchanged while splenic mRNA expression levels were up-regulated for (F) IFN- $\gamma$ , (G) NOS2, and (H) IL-10 in 6 week schistosome infected RAG<sup>-/-</sup> mice treated twice a week with 20  $\mu$ g Poly:Ic. Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



**Figure 11. Chronic TLR signaling does not result in the down-regulation of liver TNF mRNA transcription after egg deposition begins in immunodeficient mice.**

Splenic mRNA levels for (A) TNF and (B) IL-10 were upregulated in the livers of 6 week *S. mansoni* infected RAG<sup>-/-</sup> after chronic LPS stimulation. Splenic mRNA levels for (C) TNF was upregulated while (D) IL-10 transcription levels did not differ from controls in the livers of 6 week *S. mansoni* infected RAG<sup>-/-</sup> after chronic guardaquimod stimulation. Splenic mRNA levels for (E) TNF and (F) IL-10 were upregulated in the livers of 6 week *S. mansoni* infected RAG<sup>-/-</sup> with (G) TNF expression levels correlating with IL-10 transcription levels after chronic Pam3CSK4 stimulation. Splenic mRNA levels for (H) TNF and (I) IL-10 were upregulated in the livers of 6 week *S. mansoni* infected RAG<sup>-/-</sup> with (J) TNF expression levels failing to correlate with IL-10 transcription levels after chronic poly I:C stimulation.



## **Chapter 4**

**IL-1 signaling is not required for innate immune regulation after chronic  
LPS or alum stimulation**

## Introduction

Blood flukes of the genus *Schistosoma* like many parasites have a complex lifecycle involving both a mammalian definitive host and an invertebrate snail intermediate host (find ref). Schistosomes and their definitive hosts (usually human in the case of *S. mansoni*) have co-evolved over 12 million years ago [1]. These blood flukes face the challenge of navigating complex life stage transitions from one host to the next all the while evading the host immune responses. Yet at the same time they also are exploiting host immune factors as signals to coordinate their own development within the definitive host. Worms fail to develop normally in recombination activating gene-deficient (RAG<sup>-/-</sup>) mice that lack T and B cells, but development is restored after adoptive transfer of CD4<sup>+</sup> T cells [2]. Long-term administration of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), a toll-like receptor (TLR-) 4 ligand, to RAG<sup>-/-</sup> mice also restores worm development [3], indicating that innate immune signals are sufficient for parasite development to proceed normally. However, LPS is not normally present in sufficient quantities to provide the stimulus needed to promote innate immune activation raising the question of how during normal infection the innate immune response is activated for the provision of parasitic developmental cues.

The onset of oviposition by *S. mansoni* results in extensive inflammation resulting in pathological changes to the liver and intestines due to granuloma formation around the trapped eggs that has been well reported and described [4-6]. However, before egg deposition begins the development of the worms in the portal vasculature also results in both coagulative necrosis and inflammatory cell infiltration of the liver [7,8]. Through

histological analyses, we have shown that RAG<sup>-/-</sup> mice, unlike their wild type counterparts, fail to develop liver inflammation and necrosis during early infection (Chapter 2 Figure 1). However, restoring liver inflammation in *S. mansoni*-infected RAG<sup>-/-</sup> mice by long-term administration of hepatotoxic drugs restores parasite development (Chapter 2 Figure 2), suggesting that damage associated molecular patterns (DAMPs) released by dying cells, rather than PAMPs, are the physiological activators of the innate immune responses that the parasites require.

As innate immune activation by PAMPs and DAMPs both generate an inflammatory response characterized by the production of the cytokines TNF- $\alpha$  and IL-1 $\beta$  [9,10], we investigated the role of these cytokines in restoring worm development. To accomplish this, we generated RAG<sup>-/-</sup> IL-1 receptor-deficient (IL-1R<sup>-/-</sup>) double knockout mice and used neutralizing antibodies against TNF- $\alpha$  to block TNF signaling in vivo. Here we demonstrate that IL-1 receptor signaling is not required for parasite development to proceed after chronic LPS or alum stimulation. However, it is required for restoration of parasite development to proceed after TNF blockade in RAG mice suggesting that IL-1 signaling is required for regulation of pro-inflammatory responses by neutralizing antibody to TNF.

## Materials and Methods

### Ethics statement:

All animal studies were conducted in accordance with established protocols approved by the USUHS Institutional Animal Care and Use Committee.

### Experimental mice:

RAG-1<sup>-/-</sup> mice on a C57BL/6 background were originally purchased from Jackson laboratory (Bar Harbor, ME) and then bred in-house for experimental use. . RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> were generated by crossing C57BL/6 RAG-1<sup>-/-</sup> to C57BL/6 IL-1R<sup>-/-</sup> mice purchased from Jackson laboratory (Bar Harbor, ME). The RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> genotype was confirmed via PCR. All mice used in experiments were age matched.

### Parasite parameters:

Mice were infected percutaneously via tail exposure to water containing 160 *S. mansoni* cercariae (Puerto Rican strain) shed from infected *Biomphalaria glabrata* snails. At 6 weeks post infection (p.i.) mice were sacrificed. Worms were perfused from the portal system and immediately fixed in 4% neutral buffered formaldehyde. Male and female worms were counted and photographed at 20x magnification using a Nikon D80 10.0 megapixel digital camera attached to a Zeiss trinocular dissecting microscope. Worm growth was assessed by measuring the length of male worms from digital micrographs Image J software (<http://rsb.info.nih.gov/ij>), as described previously[3]. Only male worms were measured as female growth is dependent upon receiving developmental

cues from pairing with maturing males [11]. Sexual maturation of the parasites was assessed by calculating egg production per worm pair from liver egg burdens, as described previously [3]. Briefly, liver tissue was homogenized and digested in 0.7% trypsin phosphate buffered saline (PBS) solution and the released eggs were counted under a dissecting microscope.

#### Alum and LPS treatment:

RAG-1<sup>-/-</sup> or RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> mice were infected with cercariae as described above. Mice received biweekly i.p. injections of Imject Alum (Thermo Scientific, Rockford, IL) at a dose of 1 mg/mouse or ultrapure LPS, *E.coli* 0111:B4 (Invivogen) at a dose of 20 µg/mouse. Control mice received biweekly i.p. injections of PBS without calcium and magnesium. At 6 weeks p.i. mice were sacrificed and parasite parameters were determined as described above.

#### Anti-TNF-α treatment:

RAG-1<sup>-/-</sup> or RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> mice were infected with cercariae as previously described. Mice received weekly i.p. injections of Adalimumab (Abbott, Chicago, IL) at a dose of 100 µg/mouse, using PBS without calcium and magnesium as a vehicle. Control mice received weekly i.p. injections of PBS alone. At 6 weeks p.i., mice were sacrificed and parasite parameters were determined as described above.

### Anti-TNF and LPS treatment

RAG-1<sup>-/-</sup> or RAG-1<sup>-/-</sup> IL-1R<sup>-/-</sup> mice were infected with cercariae as previously described. Mice received weekly i.p. injections of Adalimumab (Abbott, Chicago, IL) at a dose of 100 µg/mouse, using PBS without calcium and magnesium as a vehicle. 24 hours and then 72 hours after receiving adalimumab the mice received i.p. injections of ultrapure LPS, *E.coli* 0111:B4 (Invivogen) at a dose of 20 µg/mouse. At 6 weeks p.i., mice were sacrificed and parasite parameters were determined as described above.

### RNA isolation, purification, and Real time PCR

RNA was isolated from the spleens and/or livers of wild type or RAG-1<sup>-/-</sup> mice. After removal, tissues were immediately placed in 1 ml RNA-BEE (Tel-Test, Friendswood, Texas), homogenized, snap-frozen in liquid nitrogen and stored at -80°C until isolation of total RNA, following manufacturer's instructions. RNA was further purified following the RNeasy mini protocol for RNA cleanup (Qiagen, Valencia, California). Purified RNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). 2 ug of RNA was used for cDNA preparation using a high capacity RNA to cDNA kit (Invitrogen, Grand Island, New York) following manufacturer's instructions. Real time PCR was performed with a MJ Research Chromo4 PTC-200 thermocycler unit (Bio-Rad, Hercules, CA) using Taqman gene expression assays and TaqMan gene expression master mix (Invitrogen) following manufacturer's instructions. Assays for the following mRNAs were performed: *rsp29*, *GAPDH*, *TNF-α*, *IL-1β*, *IL-1α*, *IL-1RA*, *IL-18*, *IL-33*, *CCL2*, and *IFN-γ*. Expression of

genes of interest was normalized to the expression of GAPDH or *rsp29* and fold changes in expression were calculated following the  $2^{-\Delta\Delta CT}$  method [12].

#### Statistical analysis

All statistical analyses were performed using GraphPad Prism Inc. version 4.0 software (San Diego, California). Significant differences between two groups were determined using a student's unpaired T-test with Welch's correction for worm lengths or a Mann-Whitney test for egg production. Significant differences between 3 or more groups for worm lengths were determined using ANOVA followed by Tukey's post test. Significant differences between 3 or more groups for egg production were determined using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. *P* values of less than 0.05 were considered significant. 3-5 mice were used per experimental group and each experiment was repeated at least twice.

## Results

### *IL-1 signaling is not required for restoration of parasite development by chronic TLR or inflammasome stimulation*

Previously, we have shown that chronic innate immune stimulation by TLR or inflammasome agonists resulted in the restoration of parasite development in RAG<sup>-/-</sup> mice. As IL-1 $\beta$  is a critical downstream mediator of both TLR and inflammasome activation [13,14] we tested whether IL-1 signaling was required for restoration of parasite development by these stimuli. In order to address this question we generated RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice by interbreeding RAG-1<sup>-/-</sup> and IL-1R<sup>-/-</sup> lines. Then groups of RAG<sup>-/-</sup>, IL-1R<sup>-/-</sup> and RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice were infected to assess parasite development. As previously reported (Chapter 2 Figure 3C and 3D), RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> did not differ from RAG<sup>-/-</sup> mice in their ability to support parasite development, with worms recovered from both groups being small in size (Figure 12A) and sexually immature (Figure 12B). Worms from IL-1R<sup>-/-</sup> exhibited normal development comparable to that observed in wild type mice, both in terms of parasite growth and egg production, consistent with the conclusion that IL-1R signaling is redundant for parasite development in animals with intact adaptive immunity. Next, we found that IL-1R deletion did not abrogate the ability of chronic LPS or alum administration to restore parasite development, as determined by measurement of parasite size (Figure 12C) and sexual maturation (Figure 12D). Together these data shows that IL-1 signaling is not redundant and nonessential for parasite development, both in immunocompetent mice and in immunodeficient mice chronically treated with TLR or inflammasome agonist.

*Blockade of TNF in RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice further stunts parasite development*

As IL-1 signaling was not required for stimulation of restoration of parasite development by LPS or alum, we hypothesized there was functional redundancy between IL-1 $\beta$  and other inflammatory cytokines, especially TNF, as these cytokines share considerable functional overlap in innate immune responses [15]. To test this hypothesis, we utilized an antibody against TNF to block TNF signaling in RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice, both in conjunction with and without chronic LPS administration. We found that TNF blockade during pre-patent infection failed to abrogate the ability of LPS administration to restore parasite development in the RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice, as determined by measurement of worm size (Figure 13B) and egg output (Figure 13C). Thus, restoration of parasite development by TLR agonist required neither IL-1 nor TNF signaling. However, we found that TNF blockade in RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice that did not receive TLR agonist resulted in significant further impairment of parasite development, with worms recovered from the anti-TNF-treated RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice being significantly smaller in size compared to the worms collected from control RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice that received vehicle alone (Figure 12A). Thus, combined IL-1/TNF blockade in the absence of TLR agonist resulted in even less favorable conditions for parasite development than observed in either RAG<sup>-/-</sup> or RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice.

*Blockade of TNF in RAG<sup>-/-</sup> mice restores parasite development similar to that of LPS administration*

The finding that anti-TNF suppressed worm development further in RAG<sup>-/-</sup>/IL-1R<sup>-/-</sup> mice was in direct contrast to our data showing that anti-TNF was sufficient to restore

parasite development in RAG<sup>-/-</sup> mice, presumably due to its ability to restore regulation of pro-inflammatory gene transcription (ref paper 1). As anti-TNF and LPS represent potentially antagonistic treatments, the former promoting control of pro-inflammatory the responses and the latter stimulating them, we tested whether there was any interference between these two treatments in their abilities to restore parasite development in RAG<sup>-/-</sup> mice. As reported previously, anti-TNF treatment alone was able to restore parasite growth and egg production in RAG<sup>-/-</sup> mice. Simultaneous treatment with both LPS and anti-TNF produced parasites that were indistinguishable in size and egg production from those obtained from animals treated with anti-TNF alone. Thus there was no evidence of an antagonistic or synergistic effects between these two development-promoting treatments.

*Cytokine transcription profiles differ in RAG<sup>-/-</sup> and RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice treated with anti-TNF*

The divergent results we obtained after TNF blockade in RAG<sup>-/-</sup> versus RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice suggested that immune responses between these two mouse strains differed after administration of anti-TNF during prepatent infection. To explore this possibility, we examined pro-inflammatory cytokine transcription levels at 24 hours (1 dose anti-TNF), one week (2 doses anti-TNF) and four weeks (5 doses anti-TNF) p.i. in the spleens of RAG<sup>-/-</sup> and RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice either treated with anti-TNF or with vehicle alone. We found at 24 hours and one week p.i. TNF, IFN- $\gamma$ , IL-1 $\beta$ , IL-18, and IL-33 splenic transcription levels did not differ from vehicle controls in the RAG<sup>-/-</sup> and RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice that received antibody to block TNF (data not shown). By four weeks p.i., however,

striking differences were seen between the mouse strains. In the RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice, where parasite development was further stunted after anti-TNF treatment, we found no differences in the splenic transcription levels of TNF, IL-1 $\beta$ , IL-1 $\alpha$ , IR-1 receptor antagonist, IL-18, IL-33, CCL2, or IFN- $\gamma$  (Figure 4A-H) in the anti-TNF treated animals versus control animals. However, in RAG<sup>-/-</sup> mice, splenic transcription levels of TNF, IL-1 $\beta$  and CCL2 (Figure 2C-E chapter 1) as well as, IL-1 receptor antagonist (Figure 5A) were down-regulated at four weeks p.i. in anti-TNF treated animals when compared to vehicle treated controls. Transcription levels for IL-1 $\alpha$ , IL-18, IL-33, and IFN- $\gamma$  in anti-TNF-treated RAG<sup>-/-</sup> mice did not differ from controls (Figure 5B-E). These striking differences between the two mouse strains suggests that blocking TNF in RAG<sup>-/-</sup> mice induced regulation of several pro-inflammatory genes, and that this anti-TNF-mediated regulation is dependent on IL-1R.

## Discussion

Early schistosome development during acute infection has been known to induce liver inflammation and necrosis in the absence of eggs [8,16]. However, in mice where adaptive immune responses are not intact there is very little in the way of liver inflammation or necrosis during prepatent infection (Chapter 2 figure 1) but induction of cell death by hepatotoxins or supplying exogenous damps to immunodeficient mice restored parasite development (Chapter 2 figure 2). This provided us with evidence that activation of the innate immune response by inflammasome signaling during normal infection could be a potential mechanism exploited by the parasite for developmental cues. As IL-1 $\beta$  is the hallmark cytokine produced in response to inflammasome activation [17-19] we wanted to further investigate the role it plays in establishing an immune environment conducive for parasite development to proceed. In order to accomplish this goal we bred RAG $^{-/-}$ IL-1R $^{-/-}$  mice. Utilizing these mice allowed us to determine the contribution of IL-1 signaling in establishing a regulated immune environment after chronic TLR or inflammasome stimulation. This mouse strain was also useful when used in conjunction with anti-TNF to look at both IL-1 and TNF signaling together as these cytokines have overlapping roles in establishing inflammatory responses.

While intact IL-1 signaling is not required for regulation of pro-inflammatory responses by chronic TLR or inflammasome stimulation it is necessary for TNF blockade to be effective as demonstrated by the failure of parasites to develop in RAG $^{-/-}$ IL-1R $^{-/-}$  mice after TNF blockade (Figure 13C). As TNF and IL-1 $\beta$  signaling have been shown to

influence each other i.e. the up-regulation of one results in the up-regulation of the other it is not surprising that TNF blockage fails to induce regulation of inflammatory cytokines in IL-1 signaling's absence (Figure 15A-H), and this data taken together indicates that in order for TNF blockade to restore *S. mansoni* development in immunodeficient animals IL-1 signaling must be intact. However, what was surprising was that worms recovered from RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice after TNF blockade were significantly smaller than those recovered from the RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice treated with vehicle control. These results suggest that while TNF blockade failed to result in transcriptional regulation of pro-inflammatory cytokines in RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice it never the less had an effect on innate responses creating an even less favorable environment for parasite development to occur in. However, the mechanisms behind how this happens remain to be elucidated.

Administering LPS and alum to RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice during pre-patent infection restored parasite development and suggested that IL-1R signaling was dispensable in creating an appropriate immune environment for parasite development in response to chronic administration of these stimuli. These results support chapter 2's finding specifically that there are multiple mechanisms by which inflammation can be regulated. Chronic LPS stimulation in particular resulted in the transcriptional regulation of not only TNF and IL-1 $\beta$  (Chapter 1, Figure 4) but IL-6 and IFN- $\gamma$  as well (Chapter 2, Figure 10). Taken together this suggests that no single cytokine is responsible for regulating inflammation in response to chronic TLR or inflammasome stimulation. It also highlights again that schistosome development in immunodeficient animals requires the

regulation of pro-inflammatory responses but that the manner in which those responses are regulated is unimportant.

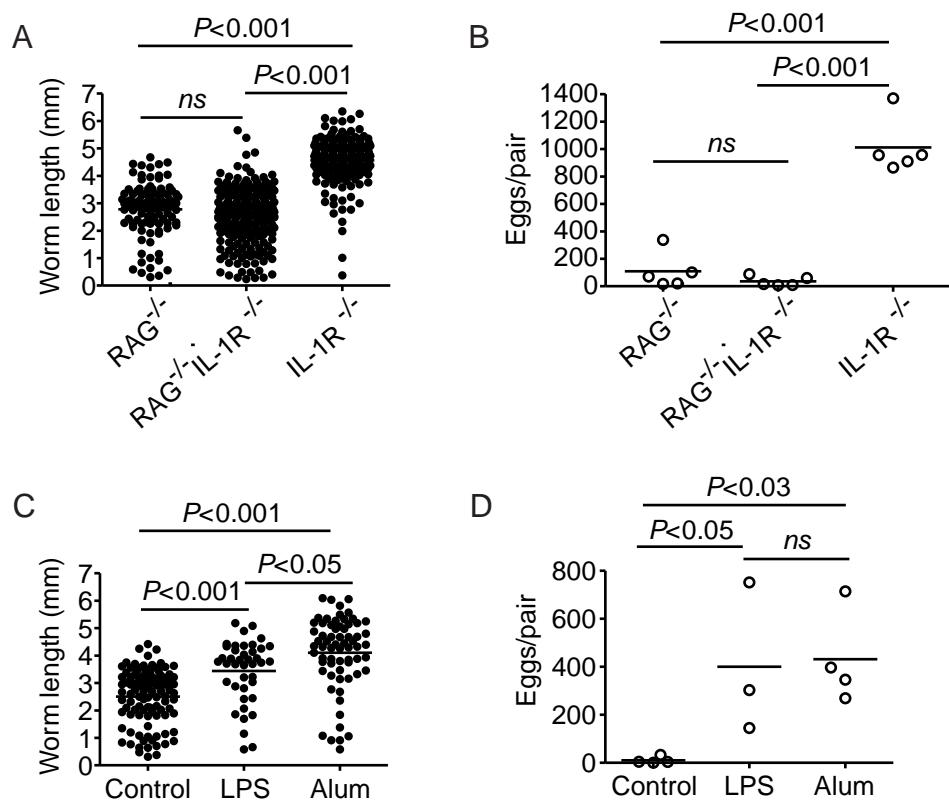
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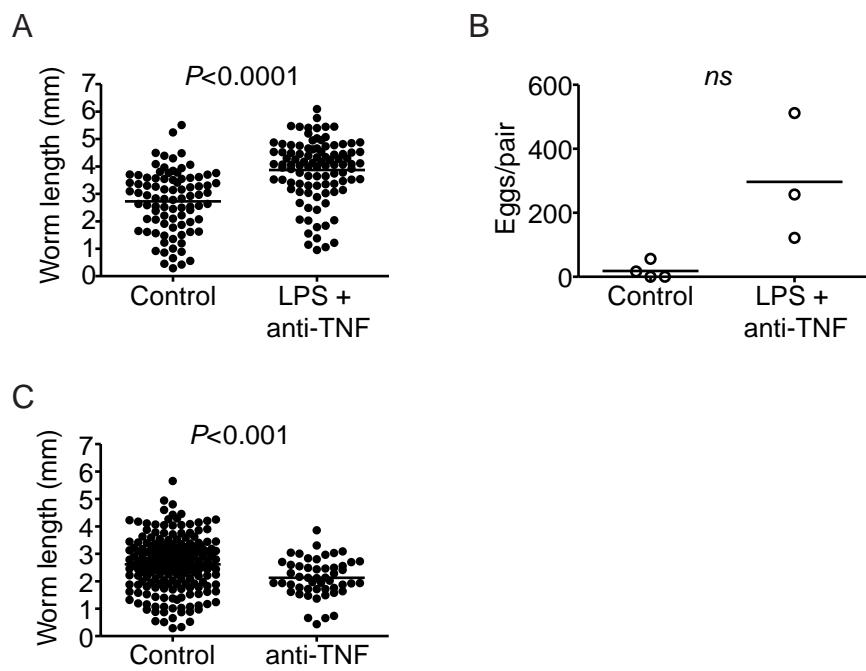
**Figure 12. IL-1 receptor signaling is not required for restoration of parasite development in immunodeficient mice after chronic inflammasome or TLR stimulation.**

Parasites development was normal in IL-1R<sup>-/-</sup> mice. (A) Length of male worms recovered and (B) egg production by schistosome pairs recovered from RAG<sup>-/-</sup>, RAG<sup>-/-</sup>IL-1R<sup>-/-</sup>, and IL-1R<sup>-/-</sup> mice at 6 weeks p.i.. Parasite development was restored after chronic TLR4 or NALP3 inflammasome stimulation in RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> during prepatent schistosome infection. (C) Length of male worms recovered and (D) egg production by schistosome pairs recovered from RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice at 6 weeks p.i. after treated twice a week either vehicle control 20  $\mu$ g LPS or 1 mg alum. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using an ANOVA followed by a Tukey's post test. P values for egg production were determined using the Kruskal Wallis test followed by a Dunn's multiple comparison post test.



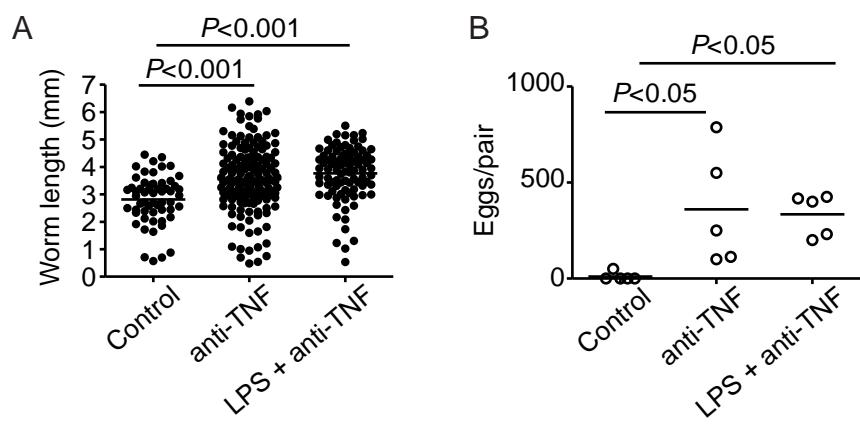
**Figure 13. Neither TNF nor IL-1R signaling are required for restoration of parasite development in RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice after chronic TLR stimulation.**

(A) Length of male worms recovered and (B) egg production by schistosome pairs recovered from RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice at 6 weeks p.i. after treatment once a week with 100 µg anti-TNF- $\alpha$  as well as twice a week with 20 µg LPS and as compared to vehicle treated controls. (C) Length of male worms from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment once a week with 100 µg anti-TNF- $\alpha$ . Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using student's T-test with Welch's correction. P values for egg production were determined using the Mann Whitney test.



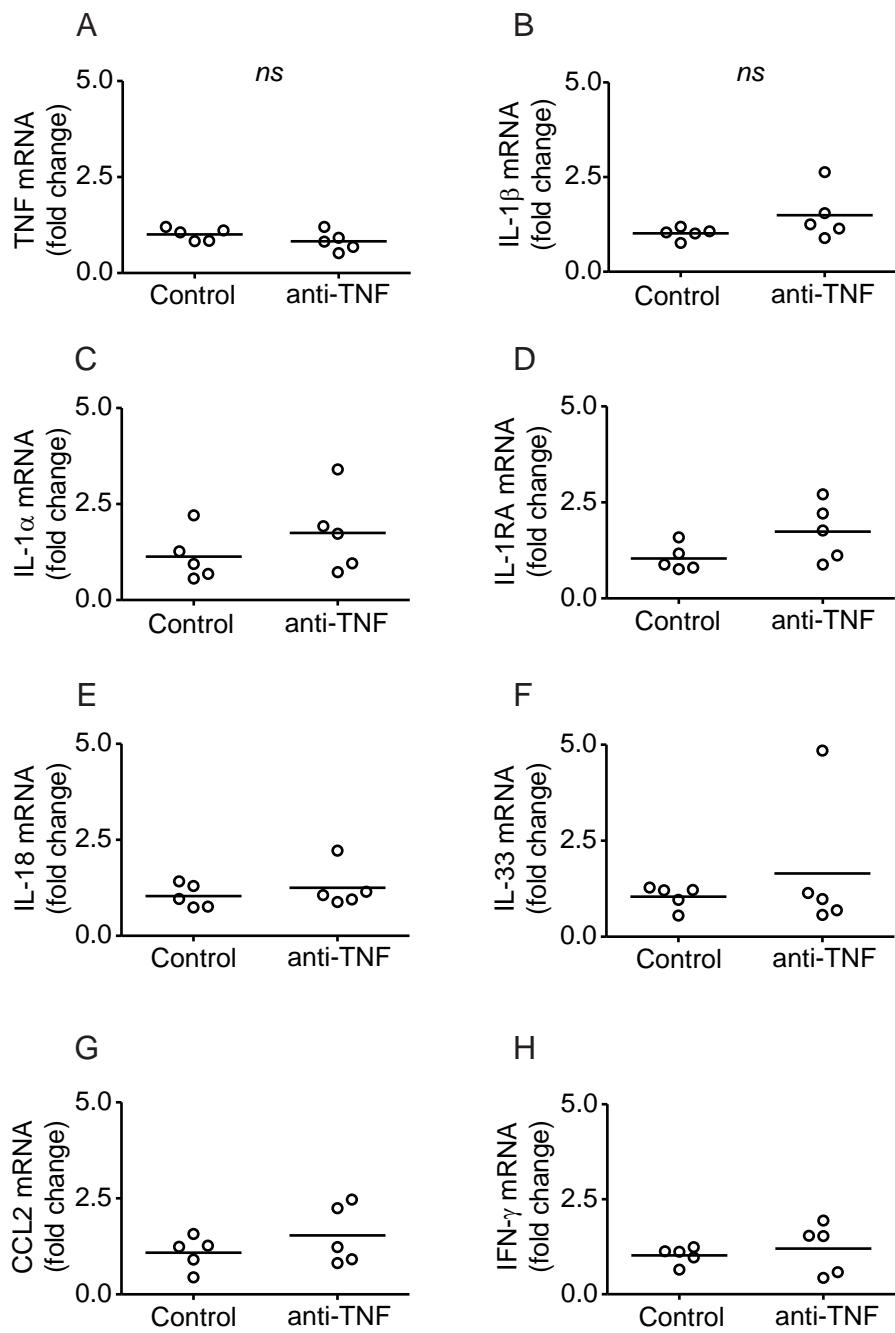
**Figure 14. TNF blockade alone is sufficient for restoration of parasite development in RAG<sup>-/-</sup> mice after chronic TLR stimulation.**

Length of male worms (A) and (B) egg production by schistosome pairs recovered from RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment once a week with 100 µg anti-TNF- $\alpha$  or after treatment once a week with 100 µg anti-TNF- $\alpha$  as well as twice a week with 20 µg LPS as compared to vehicle treated controls. Groups of 5 mice were used for each experimental condition. P values for worm lengths were determined using an ANOVA followed by a Tukey's post test. P values for egg production were determined using the Kruskal Wallis test followed by a Dunn's multiple comparison post test.



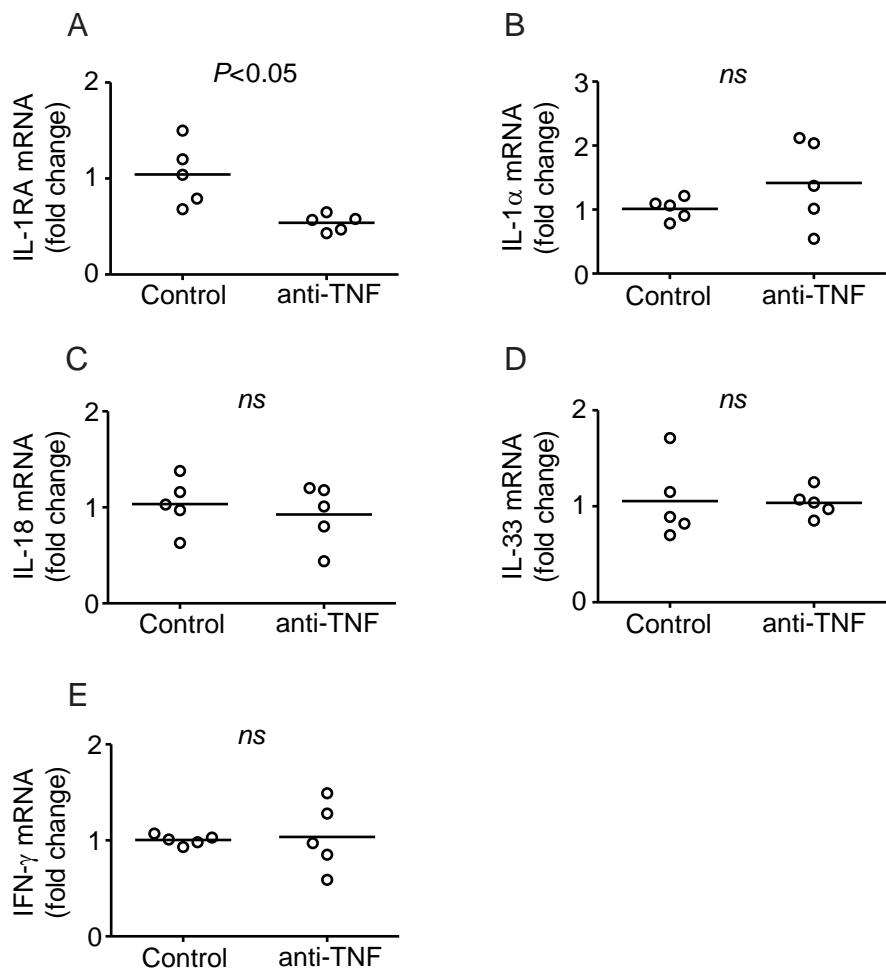
**Figure 15. TNF blockade does not result in changes to splenic inflammatory cytokine gene expression in RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice.**

Splenic mRNA levels for (A) IL-1 $\beta$  (B) TNF- $\alpha$  (C) IL-1 $\alpha$  (D) IL-1RA (E) IL-18 (F) IL-33 (G) CCL2 and (H) IFN- $\gamma$  were unchanged in 4 week schistosome infected RAG<sup>-/-</sup>IL-1R<sup>-/-</sup> mice after treatment once a week with 100  $\mu$ g anti-TNF- $\alpha$ . Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



**Figure 16. TNF blockade results in downregulated splenic IL-1 receptor antagonist (IL-1RA) in RAG<sup>-/-</sup> mice.**

Splenic mRNA levels for (A) IL-1RA were down-regulated while they remained unchanged for (B) IL-1 $\alpha$ , (C) IL-18, (D) IL-33, and (F) IFN- $\gamma$  in 4 week schistosome infected RAG<sup>-/-</sup> mice after treatment once a week with 100  $\mu$ g anti-TNF- $\alpha$ . Groups of 5 mice were used for each experimental condition. Mean values are represented by horizontal bars. P values were determined using the Mann Whitney test.



## **Chapter 5**

### **Summary and Future Directions**

## Dissertation Summary

Having established that the role of CD4<sup>+</sup> T cells in facilitating schistosome development is indirect and mediated via provision of T cell help for innate responses, the goal of the work presented here was to gain a better understanding into how innate immune responses support the development of schistosomes in RAG<sup>-/-</sup> mice. The principal questions we wished to address were (i) how does *S. mansoni* activate the innate immune response in order to facilitate its own development, and (ii) what elements of the innate immune response are required for schistosome development to occur. Having shown that chronic administration of a TLR4 agonist could facilitate parasite development, we sought to identify other and perhaps more physiologically relevant stimuli of innate immune responses that could mediate the same effects. We provided histological evidence that hepatic responses to the developing parasite were fundamentally different in RAG<sup>-/-</sup> and wild type mice, with RAG<sup>-/-</sup> mice showing little evidence of the hepatocellular necrosis and inflammatory infiltration characteristic of infection in wild type mice. Furthermore, we were able to show that by administering MSU, a product of cell death, we were able to again restore parasite development in RAG<sup>-/-</sup> mice, suggesting that in immunocompetent mice, cell death and the resultant inflammation may be important in determining the progress of parasite development. Finally, we surveyed the breadth of innate stimuli and immune modulators that either restored or impeded *S. mansoni* development and determined that improved parasite development correlated with regulation of innate pro-inflammatory responses. Among the immune modulators that restored parasite development was IL-4, a type 2 cytokine

produced by adaptive responses to pre-patent infection in immunocompetent hosts. This finding suggests that, by virtue of their ability to regulate pro-inflammatory processes, Th2 responses may be associated with schistosome development in immunologically intact hosts.

### **Timeline for *S. mansoni* development in the context of the host immune response**

The skin is the first major site for schistosome development in the definitive host, with the transformation of cercariae into schistosomula during successful invasion involving dramatic anatomical, physiological, and biochemical changes [1], including the loss of the cercarial tail, shedding of the glycocalyx, formation of the tegument, and the release of excretory secretory (ES) products from acetabular glands [2,3]. Migration of the parasites through skin via the epidermis, basement, membrane and dermis, with the eventual penetration of a dermal blood vessel or a lymphatic vessel, is estimated to take approximately 3 days [2,4]. In the circulation, schistosomula arrive in the lungs, this point in development being described as stage 1 or the “lung form” [5,6]. The parasites reside in the pulmonary capillaries, increasing 4 fold in length and with a proportionate decrease in diameter [7], these changes in morphology accomplished by redistribution of already existent cells and tissues, with no increases in mass [8] and no mitosis [6].

Migration from the lungs to the liver begins approximately 7 days post infection, with the schistosomula contracting back to their original skin morphology upon arrival in the portal circulation. Ingestion of blood begins, and is accompanied by increased O<sub>2</sub> usage

and lactic dehydrogenase activity [7]. Stages 2-6 of development take place in liver venules. Stage 2 is defined by the development of the gut at 15 days p.i. Stage 3 is defined by the development of the testes of male worms and uterus of females worms at 21 days p.i. Stage 4 is defined by the onset of gametogeny at 28 days p.i. Stage 5 is defined by the start of egg shell formation 30 days p.i. The climax of schistosome development, stage 6, is defined by the onset of oviposition at 34-35 days p.i. [5,6]. As the majority of parasite growth and development take place in hepatic venules from approximately 1 week p.i. to 5 to 6 weeks p.i. this is the time frame in which the immune responses could provide critical developmental cues.

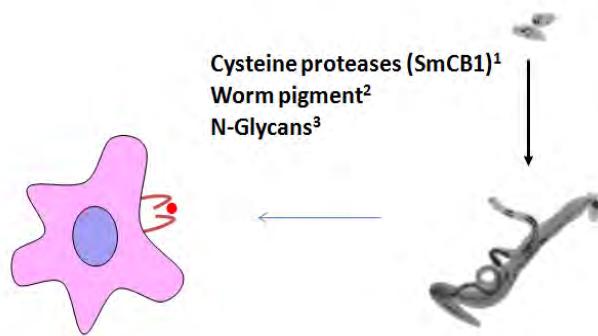
The immune responses generated early in infection in immunologically intact hosts, prior to the onset of egg production, have been described as weak Th1 like responses [9]. However, Th2 responses have also been shown to occur early in infection. Schistosomula ES components inhibit inflammatory cell recruitment, induce T-cell apoptosis and elicit human keratinocytes to produce IL-10 [2,10-13], thus potentially contributing to the overall anti-inflammatory phenotype of the host response during pre-patent infection. Migration of *S. japonicum* schistosomula through the lung capillaries also resulted in innate immune wound healing responses characterized by RELM- $\alpha$  production [14]. By four weeks post infection, IgE antibodies are produced to a *S. mansoni* cysteine protease (SmCB1) with the help of CD4 $^{+}$  T-cells and IL-4, indicating that Th2 responses do occur early in infection [15] and supporting previous reports that cysteine proteases may be pro- Th2-inducing antigens [16].. Schistosome hemozoin, also known as worm pigment, is the by-product of hemoglobin degradation and has also been shown to induce markers of alternative macrophage activation in hepatocytes [17]. We

have found worm pigment in liver sections as early as 3 weeks p.i. in wild type mice (data not shown). Schistosome glycans expressed from the beginning of infection onwards [18,19] also elicit strong Th2 responses [19] and drive alternative activation of macrophages [20]. Thus, there are multiple potential mechanisms by which schistosomes might drive the Th2 polarization of CD4<sup>+</sup> T cell responses during early infection. We therefore propose the following model (Figure 17) of how Th2 responses might be induced during infection and how these responses could potentially create an immunoregulated environment permissive for parasite development.

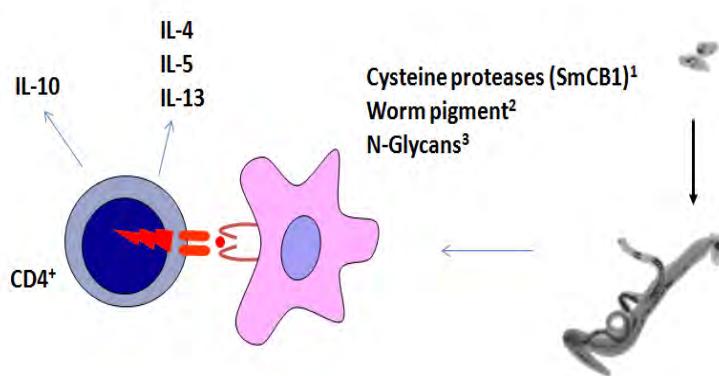
**Figure 17. Potential model highlighting early *S. mansoni* and host immune interactions during prepatent infection**

Parasite antigens interact with antigen presenting cells (APCs) (1). APCs in turn interact with CD4<sup>+</sup> T-cells resulting in a Th2 response (2). The resulting Th2 responses create an environment permissive for parasite development (3).

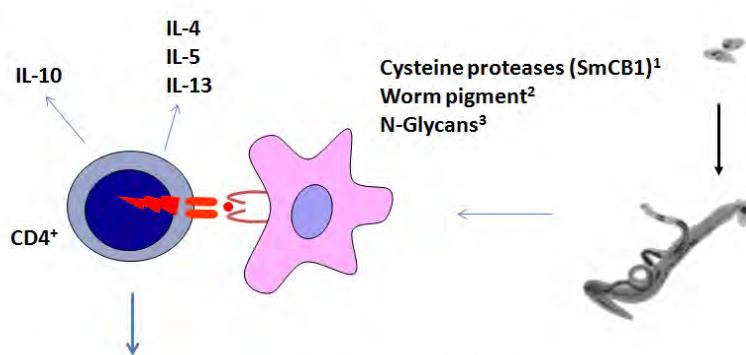
1.



2.



3.



**Creation of permissive environment for parasite development**

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## Immunological Redundancy in Parasite Development

A recurring theme in all of our studies is that there are likely multiple immunological pathways by which schistosomes can obtain the conditions necessary for development. While schistosomes have been shown to respond to human cytokines such as TGF- $\beta$  [21] and TNF [22] these signaling pathways are evolutionarily ancient and conserved among numerous eukaryotic organisms [23], suggesting that a single host cytokine alone is not responsible for restoration of parasite development in immunodeficient mice. Furthermore, with regard to the support of parasite development by adaptive responses, IL-4 is clearly not the only host factor capable of creating an environment that favors *S. mansoni* development, as parasite development is normal in mice where IL-4 or IL-4 receptor signaling is blocked [24-26]. The diverse transcription profiles generated in response to chronic TLR or inflammasome activation in RAG<sup>-/-</sup> mice also demonstrate that regulation of pro-inflammatory responses occurs in variety of ways. Importantly, there is little or no IL-4 produced in the absence of CD4<sup>+</sup> cells, and thus chronic administration of TLR and inflammasome agonists in RAG<sup>-/-</sup> does not result in regulation by inducing type 2 responses. Rather, we hypothesize that other, less clearly defined immunoregulatory states are induced under these conditions, perhaps analogous to the regulatory states found in other disease conditions such as cancer [27,28].

As both type 2 and immunoregulatory states all suffice to restore parasite development, this raises the question of what precisely schistosomes obtain from these immunological milieus. One possibility is that some destructive anti-schistosome effector mechanism is kept in check by regulatory responses. One possible candidate for such a

mechanism is the production of NO, which was previously shown to be an important correlate of immunity in animals vaccinated with irradiated cercariae. Another alternative is that the wound healing and tissue remodeling processes supported by these responses are in some way necessary for parasite development. One possible scenario is that developing schistosomes in the hepatic venules require a certain amount of niche remodeling to occur, perhaps in the form of vessel expansion to accommodate the growing parasite, and thus the angiogenesis component of wound healing may be the critical aspect of these responses for schistosomes. Inhibitors of angiogenesis have been well characterized as cancer treatments [29,30] and could be administered during early *S. mansoni* infection in wild type mice in order to further characterize the role of angiogenesis during early *S. mansoni* development. Numerous inhibitors for angiogenesis have been described in the literature, including antibodies against vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) [30,31] as well as small molecule VEGFR tyrosine kinase inhibitors [30]. The VEGF family is complex and includes 6 secreted glycoproteins and 5 receptors, making single antibody therapy difficult for cancer treatment [32]. Small molecular tyrosine kinase inhibitors make a more attractive option to block angiogenesis during early *S. mansoni* development. However, drugs like sorafenib have been shown to also induce reactive oxygen species (ROS) in endothelial cells [33] and to restore classical macrophage polarization and natural killer cell activation in hepatocellular carcinoma microenvironments [34]. Therefore the broad effects of tyrosine kinase inhibitors could confound data interpretation.

Yet another possibility is that changes in cell metabolism associated with each of these immunoregulatory states may be required in order for developing schistosomes to

meet their trophic requirements. Inflammation and immunoregulation initiate dramatic changes in cell metabolism, promoting aerobic glycolysis and increasing the availability of ATP and various precursor molecules for fatty acid and nucleotide synthesis. As parasites, schistosomes are auxotrophic for a variety of host molecules, ranging from cholesterol and fatty acids to purines, and indeed, their genomes are replete with genes that likely facilitate acquisition of these molecules from the host. Glycolysis inhibitors have been widely studied in cancer biology due to increased aerobic glycolysis occurring in tumors [35]. Two potential inhibitors that could be utilized used to test the importance of host glycolysis for schistosome development in wild type mice are 2-deoxy-glucose (2-DG) [36] and 3-bromopyruvate [37]. 2-DG inhibits glycolysis at the step of glucose phosphorylation by hexokinase, hexokinase instead phosphorylating 2-DG and resulting in the accumulation of 2-DG-P [35]. 3-bromopyruvate also interferes with glycolysis by inhibiting hexokinase activity [37]. However, both of these drugs also interfere with other aspects of cellular metabolism, resulting in cell death by other mechanisms besides ATP depletion [35], and complicating the interpretation of any effects these compounds may have on parasite development.

### **Host cells required for parasite development**

M2 macrophages are an attractive candidate for being a host factor co-opted by schistosomes and future work should focus on defining the role this cell type plays in early schistosome development. The critical role M2 macrophages play in controlling

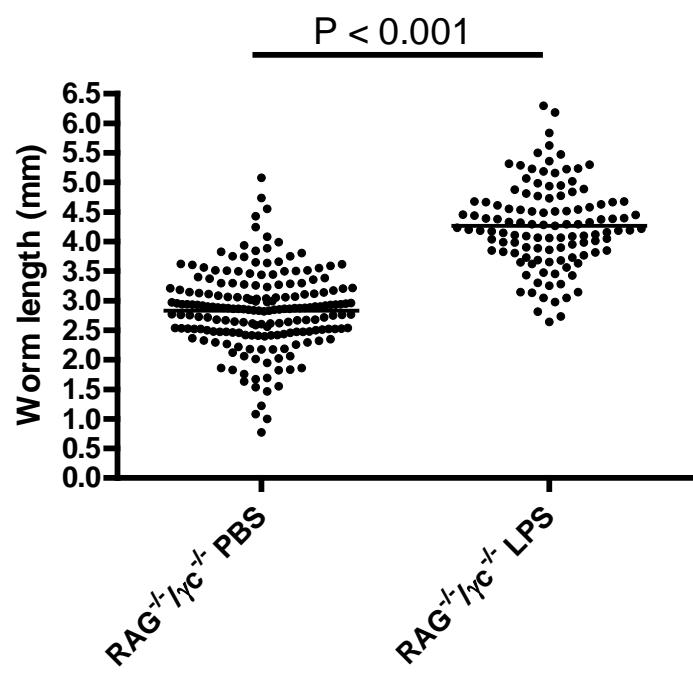
egg induced pathology has been well characterized [38-41], but their role in shaping schistosome-driven immune responses before egg deposition commences remains to be elucidated. M2 macrophage cell profiles have been well characterized by flow cytometry [42] and these tools could be utilized to characterize macrophage populations in the spleens and livers of wild type and RAG<sup>-/-</sup> mice early during pre-patent infection. M2 macrophage populations can also be adoptively transferred into RAG<sup>-/-</sup> mice during pre-patent infection, with the goal of creating an immune environment permissive for parasite development. Finally, schistosome glycans such as Lacto-N-neofucopentaose III have been shown to drive Th2 immune responses in experimental schistosome infection by inducing an alternative activation state in APCs [43,44], including macrophages [20]. Glycans may therefore be an ideal worm-derived candidate to drive type 2 responses in RAG<sup>-/-</sup> mice during prepatent infection, again with the goal of creating an immune environment permissive for parasite development.

While M2 macrophage populations are attractive candidates for shaping regulated immune states during schistosome infection, other innate cell populations also have been shown to play important roles in inducing Th2 responses during *S. mansoni* infection. Dendritic cells (DCs) in particular have been shown to be important in driving Th2 responses after exposure to either cercarial ES products [45] or egg antigens [43]. The Th2-inducing cytokine thymic stromal lymphopoietin (TSLP) has been shown to be an important cytokine involved in both the maintenance and polarization of Th2 responses by DCs [46,47]. Although parasite development was normal in TSLPR<sup>-/-</sup> mice [48], TSLP and IL-4 are host factors that could potentially be co-opted by schistosomes to provide developmental cues. Provision of exogenous TSLP to RAG<sup>-/-</sup> mice would test whether

TSLP can induce an environment conducive to parasite development, as we established for IL-4. If TSLP administration restored parasite development in RAG<sup>-/-</sup> mice, further experiments could be conducted using RAG<sup>-/-</sup> common gamma ( $\gamma$ c) chain knockout ( $\gamma$ c<sup>-/-</sup> RAG<sup>-/-</sup>) mice to tease out the contribution of type 2 innate lymphoid cells (ILC2s) in this process, as this cell population also produces Th2 cytokines in response to TSLP [49]. However, ILC2s are unlikely to play a non-redundant role in restoration of parasite development. Because we previously found that LPS could restore parasite development in  $\gamma$ c<sup>-/-</sup> RAG<sup>-/-</sup> mice (Figure 19). These findings suggest that  $\gamma$ c chain cytokine-dependent innate cells are not required for the regulation of innate immune responses critical for parasite development to occur.

**Figure 18. Chronic stimulation of TLR4 with bacterial LPS facilitates *S. mansoni* development in  $\gamma$ c<sup>-/-</sup>RAG<sup>-/-</sup> mice.**

(A) Length of male worms recovered from  $\gamma$ c<sup>-/-</sup>RAG<sup>-/-</sup> mice at 6 weeks p.i. after treatment twice a week with either vehicle control or LPS. Groups of 5 mice were used for each of the experimental conditions. P values for worm lengths were determined using student's T-test with Welch's correction.



## Concluding Remarks

Davies and McKerrow suggested that delaying development in immunodeficient hosts could provide a selective advantage to the parasite allowing it to prolong its survival [50]. The work put forth in this dissertation adds further support to what Davies and McKerrow originally proposed. It is intriguing to think that *S. mansoni* might have evolved to recognize host immune regulation early in development before sexual maturation in order to ensure its long term survival. Fully developed worms can live in the mesenteric vasculature for years [51] releasing eggs to ensure that their genes survive. If they are in the right immune environment i.e. one where there is appropriate granuloma formation to ensure the passage of the egg into the lumen of the intestine and to protect the host from the egg toxins as well there is a better chance of this occurring. If excessive pathology due egg production can be mitigated this allows the host to survive and again increases the chances of the eggs escaping to be passed with the feces and continue the life cycle. Thus, host immune responses as developmental cues makes evolutionary sense and gives the parasite a selective advantage, providing an elegant mechanism to ensure their own survival. A better understanding of early host schistosome interactions could provide useful targets for disrupting schistosome infections by improved treatments or vaccine development.

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